THE EFFECT OF HEAVY METAL CONTAMINATION ON ESTUARINE BENTHIC FAUNA AT VARYING LEVELS OF BIOLOGICAL ORGANISATION

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THE EFFECT OF HEAVY METAL CONTAMINATION ON ESTUARINE BENTHIC FAUNA AT VARYING LEVELS OF BIOLOGICAL ORGANISATION

by

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The effect of heavy metal contamination on estuarine benthic fauna at varying levels of biological organisation

Abstract

Sarah Alicia Michelle Perryman

Biological monitoring techniques, selected to cover increasing levels of biological organisation (cellular, individual, population and community), were compared and contrasted to determine the relative sensitivities of these approaches to heavy metal contamination. The study was centred on the Fal Estuary in Cornwall, an ideal experimental field site with well documented heavy metal contamination resulting from the long-term discharge of waste from a local tin mine. Five creeks leading from the estuary differ dramatically in the concentrations of heavy metals in the sediments, but little in natural environmental variables (sediment granulometry, salinity etc.).

The contaminant induced damage was evaluated at the cellular level in *Mytilus edulis* by the **Neutral Red Assay** to detect lysosomal membrane damage. Parameters of the energy budget of this species were determined to estimate **'Scope for Growth'** of individuals, along with a **Larval Survival assay** to establish their reproductive and developmental success. At the population level, **Cohort Growth studies** were undertaken using the polychaete *Nephtys hombergi*. At the community level, data from an extensive three year survey of **benthic macrofauna structure** were analysed. In addition to investigating biological parameters, contaminant levels in sediment and tissue were determined using Atomic Absorption Spectrommetry.

All techniques, except the population level, detected the extreme pollution of the most contaminated creek, Restronguet, but did not accurately reflect the known metal gradient. However, community studies established distinct macrofaunal assemblages between both sites and years. Techniques were compared and validated in a novel approach using the program **BIOENV**, with the multivariate analysis package **PRIMER** (Plymouth Routines in Multivariate Ecological Research). The cellular assay correlated reasonably well against the heavy metal levels, the larval survival assay correlated well to the community structure. However, the community structure approach was the most sensitive and relevant monitoring method to determine the long-term contamination of the Fal Estuary.

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Abbreviations

R	Restronguet
M	Mylor
P	Pill
J	St. Just
E	Percuil

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Author's declaration

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Relevant scientific seminars and conferences were attended, at some of which work was presented:

* Oral presentation to Department of Biological Sciences, May 1994. entitled: The effect of heavy metal contamination on estuarine benthic fauna at varying levels of biological organisation

* Attendance at Marine Biodiversity Conference, University of York, September 1994.

* Oral presentation to C.S.I.R.O. Laboratory, Perth, Australia, October 1994, entitled: The effect of heavy metal contamination on estuarine benthic fauna at varying levels of biological organisation

* Poster presentation at S.E.T.A.C. (Society for Environmental Toxicology and Chemistry) Conference, September 1995, Plymouth, U.K., for which I was awarded a Student Prize for my presentation, entitled: The effect of heavy metal contamination on estuarine benthic fauna at varying levels of biological organisation

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Signed.....

Date.....17th April '97.....

CHAPTER 1

General Introduction

"In the face of man's rapidly increasing activities which deform and even destroy ecosystems, it is of paramount importance to develop and refine an effective instrumentarium for determining the degree and type of man's impact and for analysing its manifestations at various sub- and supra- individual levels."

Otto Kinne (1988)

MARINE POLLUTION MONITORING

Background

The Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP 1995) defined **pollution** as 'the introduction by humans, directly or indirectly, of substances or energy into the marine environment (including estuaries) resulting in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities, including fishing, impairment of quality for use of seawater and reduction of amenities'; very much an anthropocentric interpretation, whereas Moriarty (1983) defined it as 'substances that occur in the environment at least in part as a result of man's activities, and which have a deleterious effect on organisms'; a more ecocentric definition. Distinct from pollution is **contamination** which GESAMP (1995) defined as 'an increase in background concentration of a chemical or radionucleide'; that is, pollution is a result of contamination.

The purpose of marine pollution **monitoring** as defined by a U.S. interagency marine pollution committee is 'to obtain time-series data for detecting significant changes, to provide timely warning and other advice to management so appropriate action may be taken' and by GESAMP (1995) as 'the observation of a variable over space or time in order to determine the condition or state of the ecosystem'.

Marine pollution monitoring has seen advances in attitudes and changes of emphasis over recent decades. In the 1960s and 1970s marine pollution studies mainly focused on detecting the dangers posed to human health from contaminants in marine food products *e.g.* mercury poisoning, in Minamata, Japan (Clark 1992). During the 1980s the emphasis widened to include preservation of marine ecosystems in their own right and not simply to limit effects to humans (Smith *et al.* 1981, Ward *et al.* 1984, Ritz *et al.* 1989). Efforts have recently concentrated on defining more ecologically relevant techniques for assessing the effects of contaminants on ecosystems (Calow 1992, Depledge & Hopkin 1995) and regulatory practices have demanded development of evermore sensitive methods for assessing toxicity (Lowe *et al.* 1992, Pulsford *et al.* 1994, Dixon & Pascoe 1994). Whereas the sole aim previously was to repair damage, the requirement now is to enhance, protect and preserve the ecosystem (Chapman 1995).

Changes in regulation have also resulted in increasing emphasis on ecology, reflected in the greater profile of ecotoxicology over toxicology as extrapolation from laboratory findings to the field gains importance (Calow 1992, Forbes & Forbes 1994, Depledge & Hopkin 1995). Simultaneously there has been an increase in public awareness of environmental

matters, pressure for conservation and the need for effective management including water pollution assessment.

More specialised and sensitive techniques have revealed that the effects of contaminants in natural ecosystems are extremely complex, and often unpredictable (Sheehan *et al.* 1984, Depledge 1994). Present marine pollution problems often involve mixtures of chemicals rather than a simple toxicological response to a single contaminant. In some circumstances this may result in sub-lethal effects difficult to detect, and even when detectable the causative agents may not be readily apparent. The demand for ever more sensitive techniques has fuelled a debate over the ecological relevance of extrapolating from results gained from laboratory studies to the environment. At the same time the sensitivity of any method may not be equivalent to accuracy (Chapman 1995).

Chemical versus biological monitoring.

Pollution monitoring in industry has been dominated by the use of chemical analyses of contaminants driven largely by the use of chemical criteria in pollution legislation (Stebbing *et al.* 1992). Such data have relevance only to environmental quality when related to their biological implications because they do not indicate the bio-available fraction to which the biota will respond, *i.e.* the mere presence of contaminants does not necessarily imply pollution, as defined by GESAMP. Biological techniques provide a response to the total contaminant load whether identified and quantified or not. An acute pollution incident could also be easily missed by intermittent chemical monitoring, but have a prolonged effect on the biological system (see Figure 1.1). However, observation of any biological effect does not indicate the cause because the vast majority of tests are generalised rather than contaminant specific.

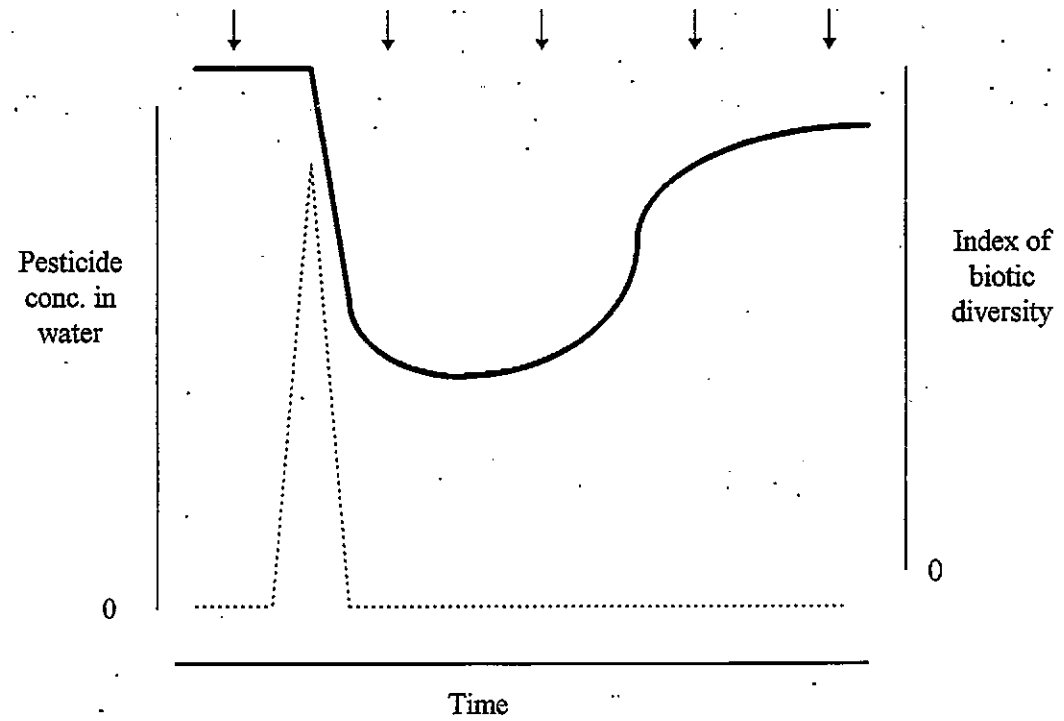

Figure 1.1 Diagram representing a pollution incident with pesticide in a river system.

Intermittent monitoring is indicated by the series of arrows. It is unlikely that infrequent chemical monitoring of the chemical composition (dashed line) would detect the incident; however, monitoring of the biota (continuous line) is likely to indicate that the incident had occurred since the timescale of its effect is much longer than the inter-monitoring period (After Goldsmith 1991).

Monitoring at various levels of biological organisation

Effects of pollutants can be detected at several levels of biological organisation, ranging from the level of the whole ecosystem successively to the sub-cellular and molecular (see Figure 1.2).

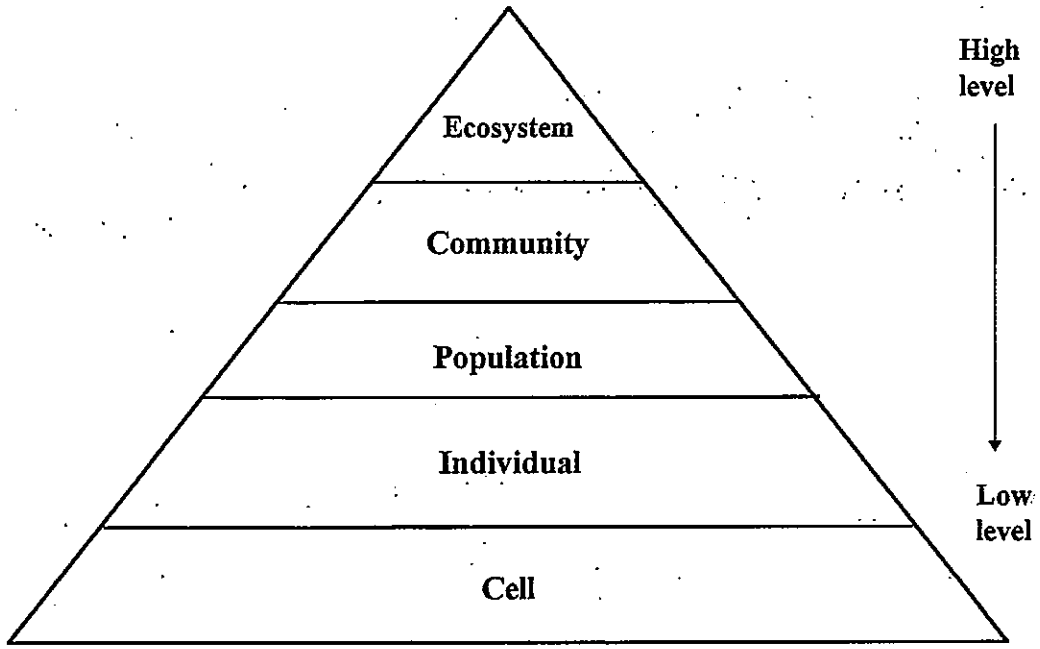

Figure 1.2 Diagrammatic representation of the varying levels of biological organisation.

Various techniques can be employed to assess the condition of any system at each of these levels. In the past, community structure assessment has been the most popular (Warwick 1993), but ecotoxicological approaches focusing on lower levels of organisation are increasingly being adopted. Some techniques and monitoring objectives aim to anticipate deleterious effects and others to quantify pollution damage retrospectively, emphasising both sublethal and lethal effects. For predictive purposes monitoring techniques must ultimately provide maximum significant information for decision making with minimum cost, hence the importance of understanding the mechanisms, links and relationships between each of the techniques targeted at the various levels.

There is a range of techniques at each of the levels:

Ecosystem

Ecosystem monitoring, involving alterations in primary productivity, nutrient cycling and food web patterns, and changes in successional patterns, is generally considered unmanageable (Warwick 1993).

Community

Studies at the community level of organisation have been the popular approach to biological monitoring (Warwick 1993). The effects are more commonly assessed through field observations (*e.g.* Olsgard 1993, Somerfield *et al.* 1994a & b) rather than by experimental manipulation, though there are exceptions. Austen *et al.* (1994) showed responses of meiofauna to chemicals in microcosms; however, these have limitations and their primary role can only be supportive of field studies (Carpenter 1996). At this level changes can be detected by examining a range of parameters such as species composition, dominance, indicator species (*e.g.* organic pollution indicated by prevalence of a capitellid worm, Pearson & Rosenberg 1978, Anger 1977); species diversity changes; log-normal distribution of individuals per species (Williamson 1987); pollution-induced community tolerance (PICT) (Blanck *et al.* 1988, Millward & Grant 1995); and benthic community structure changes involving *k*-dominance curves (Warwick *et al.*1987), ABC curves (Warwick 1986) and various multivariate methods (Gray *et al.* 1988, Gee *et al.* 1992, Clarke & Warwick 1994).

Criticisms of such tests include the lack of decisive endpoints, limited accuracy, lack of sensitivity, lack of cost-effectiveness in terms of labour and time input (Cairns *et al.* 1993),

the advanced level of taxonomic expertise required (Depledge & Hopkin 1995), the difficulty in distinguishing between natural and pollution-induced effects, and the fact that once a pollutant is evident at the community level the damage has already been done to the environment. Advocates of this level of approach have countered these criticisms; for instance, there is no evidence that experimental monitoring at the community level is less sensitive than other methods (Warwick 1993), and that as it is the environment that is the focus of concern, this is the most ecologically relevant level of study. This level will be covered in Chapter 3.

Population

Monitoring at population levels has been the most neglected area of research (Gray 1979, Depledge & Hopkin 1995). This approach is also generally conducted in the field. Methods of detecting pollutant effects at this level may include loss of individuals, alteration in growth rates (Hartnoll & Bryant 1990), reduced fecundity (Kirkegaard 1978), change in age structure (Kendall 1987b), altered gene pool, and disturbance of biological rhythms.

However, it has been criticised for being labour intensive, the lack of clear endpoints, subjectivity, and again the difficulty in distinguishing between pollution-induced and natural effects. This approach will be covered in Chapter 4.

Individual

Monitoring at this level is typically laboratory based. Approaches essentially include physiological energetics in terms of 'scope for growth' (Bakke 1988, Widdows & Johnson 1988) and reproductive success assays such as larval survival studies (McFadzen 1992, Williams 1992). Such whole organism studies can show up inter-individual differences in

pollution responses. A resulting selection pressure could cause pollution-induced ecological change and hence a significant biological effect on a system through restructuring of communities or changed genotypic distributions in a population (Blanck *et al.* 1988).

Physiological monitoring has been criticised for its lack of specificity in relating to specific substances at known concentrations (Depledge & Hopkin 1995), and the initial high cost of equipment and complex methodology. This level of approach will be covered by two techniques described in Chapters 5 and 6.

Cellular

The first response of organisms to a stress takes place at cellular and molecular levels of target organs and tissues before effects become visible at higher levels of biological organisation (GESAMP 1995). At cellular and molecular levels there are many pathological changes and biomarkers that have been identified that signify contaminant exposure. These have been developed for diagnostic screening indicators, for instance, DNA strand breaks, chemically induced oxidative stress, pathological changes of intracellular membranes of lysosomes and metallothionein production. It has been suggested that such biomarkers are the most powerful tools for investigating pollutants *in situ* (Depledge & Hopkin 1995). Some are non-specific, for example, behavioural and physiological biomarkers. Some are more specific, for example metallothioneins (to cadmium, copper and zinc) and stress proteins (to thermal pollution, tributyltin compounds, ultra-violet radiation, copper and poly-aromatic hydrocarbons). Other tests include morphological biomarkers, for example imposex changes in the dog whelk, *Nucella lapillus* (Gibbs & Bryan 1986), and pathological and disease biomarkers such as hepatic lesions in flounder (Carr *et al.* 1991),

and deformities in smelt, *Osmerus eperlanus* (Pohl 1990), and fish immune system alterations (Pulsford et al. 1994).

However, these techniques have been criticised for being expensive and for their questionable relevance to the environment (Depledge & Hopkin 1995). This level of approach will be covered by Chapter 7.

An ongoing debate - relevance and sensitivity

There has been an extensive and ongoing debate in recent years over which are the most environmentally relevant and sensitive methods for detecting pollution at the different levels of biological organistion:-

Some claim that the most powerful tools for investigating pollutants are 'biomarkers', that is those tests at the lowest level of organisation (Depledge 1994). However, these tests are criticized for their often unknown ecological significance, their minimal to non-existant laboratory to field verification (Richardson & Martin 1994, Depledge 1994), their lack of feasibility for extrapolation to community levels (Warwick 1993), and whether or not the chosen test organism is representative of other species of the community in question (Underwood & Peterson 1988). For instance, detecting changes of a particular biomarker at the molecular level in the tissue may have little significance for the health and survival of the whole individual. Alterations at the level of the individual may not be apparent at the population or community levels (Moriarty 1983) *e.g.* a rise in mixed function oxidase (MFO) in the livers of fish taken from a polluted area may signify pollutant exposure, yet the fish may continue to grow and reproduce normally, therefore the rise in MFO could be

viewed as an acclimatization process to altered environmental conditions rather than a manifestation of an injury (Depledge & Hopkin 1995). It is debatable as to whether methods at low levels are worthy of their claim to be early warnings ('ecological canaries') of imminent pollution at higher levels of organisation. Also results of laboratory based tests may be non-representative of the field situation due to recruitment of individuals from outside the area.

Others say that the most ecologically relevant and sensitive measurements are those higher level tests that describe changes in community structure or function (Kelly & Harwell 1989, Warwick 1993, Gray 1995). However, such measurements have been criticized for lacking any predictive powers (Depledge & Hopkin 1995), *i.e.* being retrospective, relying on lethal effects for their expression and so being of limited use because the ecosystem or community assemblages are already damaged. But effects could be sublethal, *e.g.* reduced fecundity decreasing recruitment and thus reducing input of the population to the community. Nevertheless, community studies have historically lacked any perception of underlying processes determining the observed change in community structure (Attrill & Depledge in press).

Relative sensitivities and reliabilities of the different methods for detecting pollution have been debated in attempts to determine which methods might be best for given purposes in terms of which tests actually detect pollution, at what level of sensitivity, and how early the method detects the pollution (Underwood & Peterson 1988).

Recent work aiming to understand the links between the levels

The above debate has led to reviews of techniques available, and, in particular, a consideration of the relevance of each method, *e.g.* GEEP (Group of Experts on Environmental Pollution) workshops; The Aquatic Science Association and their conference on Water Quality and Stress Indicators in Marine and Freshwater Ecosystems (Sutcliffe 1994); the SETAC (Society for Environmental Toxicology and Chemistry) conference on Unifying Themes in Environmental Chemistry and Toxicology (1995); Nipper *et al.* (1996), Attrill & Depledge (in press), Hawkins *et al.* (1994), Slobodkin (1994). Particular emphasis has been on the importance of linking the various approaches used at the different levels of biological organisation.

There has been relatively little direct work done to actually compare techniques with calls for this to be remedied. Cajaraville *et al.* (1995) commented on such a lack of information: "to our knowledge there has been little attempt to statistically correlate cellular assays with populations, communities and ecosystem. This work is highly recommended". Chapman (1990) also points out that biomarkers have great promise and could provide early warning of adverse effects, as well as exposure, but that there is no link to higher level effects. Attrill & Depledge (in press) indicate that the target for future investigations should be to understand mechanisms linking levels of biological organisation throughout the levels otherwise subsequent predictions or management could fail. This is important in terms of which level of organisation would provide the most sensitive and robust method of assessment.

Comparing the levels

In no case have authors covered all levels of biological organisation and instead most comparisons are by scientists working at one of the levels who then compare their data with studies by others. The conclusion that methods good at the lower levels are not so relevant at the higher levels (Nipper *et al.* 1996, Hawkins *et al.* 1994, Cairns *et al.* 1993, Johnson *et al.* 1994, Kloepper-Sams *et al.* 1994) has fuelled a 'so what?' question with respect to lower level tests. Increased complexity at the population and community levels (in particular of competition, predation and nutrient interactions) means that data based on single species should not be extrapolated to higher levels of biological organisation (Cairns *et al.* 1993). In contrast, other studies show that effects are apparent at more than one level and claim 'early warnings' can be provided (Baumann *et al.* 1990, Sanders *et al.* 1991, Munkittrick *et al.* 1994, Forbes & Forbes 1994). Many studies ultimately suggest that no single approach is satisfactory and emphasise the importance of an approach of integrated assessment involving several methods, each focusing on a different level of biological organisation. (*e.g.* Depledge & Hopkin 1995, Bayne *et al.* 1992, Underwood & Peterson 1988). However, most recent work has concentrated effort in developing ever more sensitive sub-individual level tests (Dixon & Pascoe 1994, Pulsford *et al.* 1994).

GEEP highlighted the need to evaluate and compare means for measuring the effects of chemical contaminants on marine organisms (Bayne *et al.* 1988). This resulted in a series of comparative workshops on the effects of pollutants. The first one was the Oslo workshop (Bayne *et al.* 1988), the second extended the approach to tropical seas in the Bermuda workshop (Addison & Clark 1990), and finally the Bremerhaven workshop on biological effects of contaminants in the North Sea (Stebbing *et al.* 1992b). They were of an increasingly large scale and complexity and deployed techniques from the molecular to the

community levels of biological organisation, aiming to relate the results to a range of chemical measurements of tissue, water and sediments. None of these workshops was targetted towards heavy metal contamination, being based mainly on organic pollution gradients (with complications of industrial wastes in the Bermuda study). They employed a multitude of scientists from various countries.

The workshops found that many techniques at various levels responded to contamination differences, the oyster embryo bioassay and MFO activity in Dab liver being particularly recommended and reliable, but some did not relate to the chemical data or show any significant response. They concluded that, despite the requirement by environmental managers, there is no single biological measurement that will indicate the effects of pollution. A suite of techniques is preferable to provide different types of information, from the specificity of some biochemical determinations to the integrated recognition of ecological effects that can be gleaned from effects on community structure (Stebbing *et al.* 1992a).

Estuaries and heavy metal impact

An estuary may be defined as a partially enclosed body of water having free connection with the open sea and influenced by freshwater runoff from land drainage (Bowden 1967, Pritchard 1967). From a chemical point of view an estuary may be defined simply as a region in which sea water is diluted with freshwater from land drainage (Burton 1976). The resulting sharp gradients in physico-chemical parameters of salinity, temperature, dissolved oxygen, pH and suspended matter give rise to complex behaviour of chemical constituents like heavy metals (Turner 1990). Typically prolific in estuaries, suspended matter can act as

a scavenger for metals in solution, where they are removed from solution and deposited in the sediment, often in concentrations many times higher than in the overlying water. In contrast, metals may be lost from particulate material to solution by desorption processes. Heavy metals in the estuary system therefore may be either dissolved in suspended matter, in the interstitial water or in deposited sediment. Sediments with different grain size or organic matter content will possess different heavy metal contents, mainly in the finer clay and silt fraction.

The physical and chemical nature of estuaries and behaviour of metals

Heavy metals have been defined from atomic chemistry theory on the basis of their position in the periodic table (Burrell 1974). Biochemists consider trace elements, which include some heavy metals, as those essential to living organisms for metabolism in extremely small concentrations (Turner 1990). However, some treat these terms flexibly, especially when concerned with pollution connotations. Estuaries are particularly subject to heavy metal contamination, where incoming natural and anthropogenic inputs can become trapped. They act as sinks for river derived sediments consequently becoming enriched with various components. GESAMP (1990) stated that mine tailings are of particular concern.

Heavy metals and effects on organisms

At suitable concentrations metals such as iron, copper and zinc are essential for living organisms, but most metals whether essential or not are potentially toxic to living organisms above threshold concentrations (Bryan 1976). However, metal concentrations are a poor guide to their potential toxicity to benthic species (Bryan & Langston 1992). They may be modified by mobilisation to interstitial water, their chemical speciation is variable, they may be transformed (*e.g.* methylation of arsenic), they may be preferentially bound to sediment

components such as organic matter and oxides of iron; and there may be competition between chemically similar metals such as copper and silver, zinc and cadmium, for uptake sites in organisms. These processes are further influenced by physical factors such as salinity, oxygen, pH and the stability of the sediment (Bryan & Langston 1992). Biological factors such as bioturbation (Decho 1990), mucus (Howell 1982), and trophic relationships between species can further influence uptake of metals by benthic organisms. Also, the effects can be ameliorated by metal tolerance mechanisms in some species or by the evolution of tolerant strains (Bryan &Langston 1992).

Aims

The primary objective of this work was to compare the effects of heavy metal contamination on estuarine benthic organisms at varying levels of biological organisation and to determine and compare the relative sensitivities of selected approaches. The project aimed to provide an overview of the use of field data from communities and populations in ecological assessment and how these methods relate to other techniques chosen at the individual and cellular response levels. This study examines the debate referred to, investigating which approach is the most sensitive, relevant and hence appropriate to use.

The following techniques were selected for evaluation:

Community

Analysis of benthic estuarine faunal community structure of the intertidal mud flats.

Population

Frequency / size analysis of a population of the polychaete worm *Nephtys hombergi*.

Individual

(i) Larval survival of *Mytilus edulis*

(ii) Physiological measurements on *M. edulis* in terms of scope for growth.

Cellular

Measurements of intracellular membrane damage of lysosomes in *Mytilus edulis*.

The techniques listed decrease in terms of levels of biological organisation. They were simultaneously deployed on the same contamination gradient and were related to chemical data for the sediment. However, it was not intended to investigate the specific heavy metal gradient, which is well documented (Bryan & Gibbs 1983, Somerfield *et al.* 1994a) although sediment metals were determined for a current comparison.

A literature review of each technique opens each relevant chapter (3 to 7). However, the following chapter describes experimental sites in addition to general materials and methods pertinent to each technique. A chapter is devoted to each of the five techniques, and then they are compared and evaluated in an overview chapter (Chapter 8) with a discussion of the advantages and disadvantages of each technique. Additional abiotic data are presented (Chapter 2) as an aid to interpreting the subsequent biological data.

CHAPTER 2

Sampling Sites, General Materials And Methods, Description Of Environmental Variables

INTRODUCTION

Before proceeding with the five techniques under investigation (Chapters 3-7) I will first describe the Fal Estuary environment with respect to the heavy metal gradient present in the estuary sediments, establish the lack of variation in various other environmental variables and so provide justification for the suitability of the experimental location. This chapter includes results of the determination of environmental variables as baseline data.

The Fal Estuary environment and mining history

Geomorphology and ecology

The present work was undertaken in the Fal Estuary (Figure 2.1), one of a number of rias (drowned river valleys) along the south west coast of England. Like the Yealm, Kingsbridge and Helford estuaries it is generally fully saline (Farnham & Bishop 1985). The estuary is a designated site of special scientific interest (SSSI), and is nationally important for a variety of bird life such as the little egret and red shank. There is a voluntary Marine Conservation Area of *c.* 600 hectares including the St. Just and Percuil inlets in the south east which includes a bank of maerl, a calcified seaweed (*Phymatolithon*

calcareum) of considerable interest (Williams 1994). Elsewhere in the Fal dead maerl has been dredged for use as a fertiliser. The maerl beds typically support a diverse community of invertebrates, fish and epiphytic algae. The west side of the estuary is not so diverse due to mining derived contaminants.

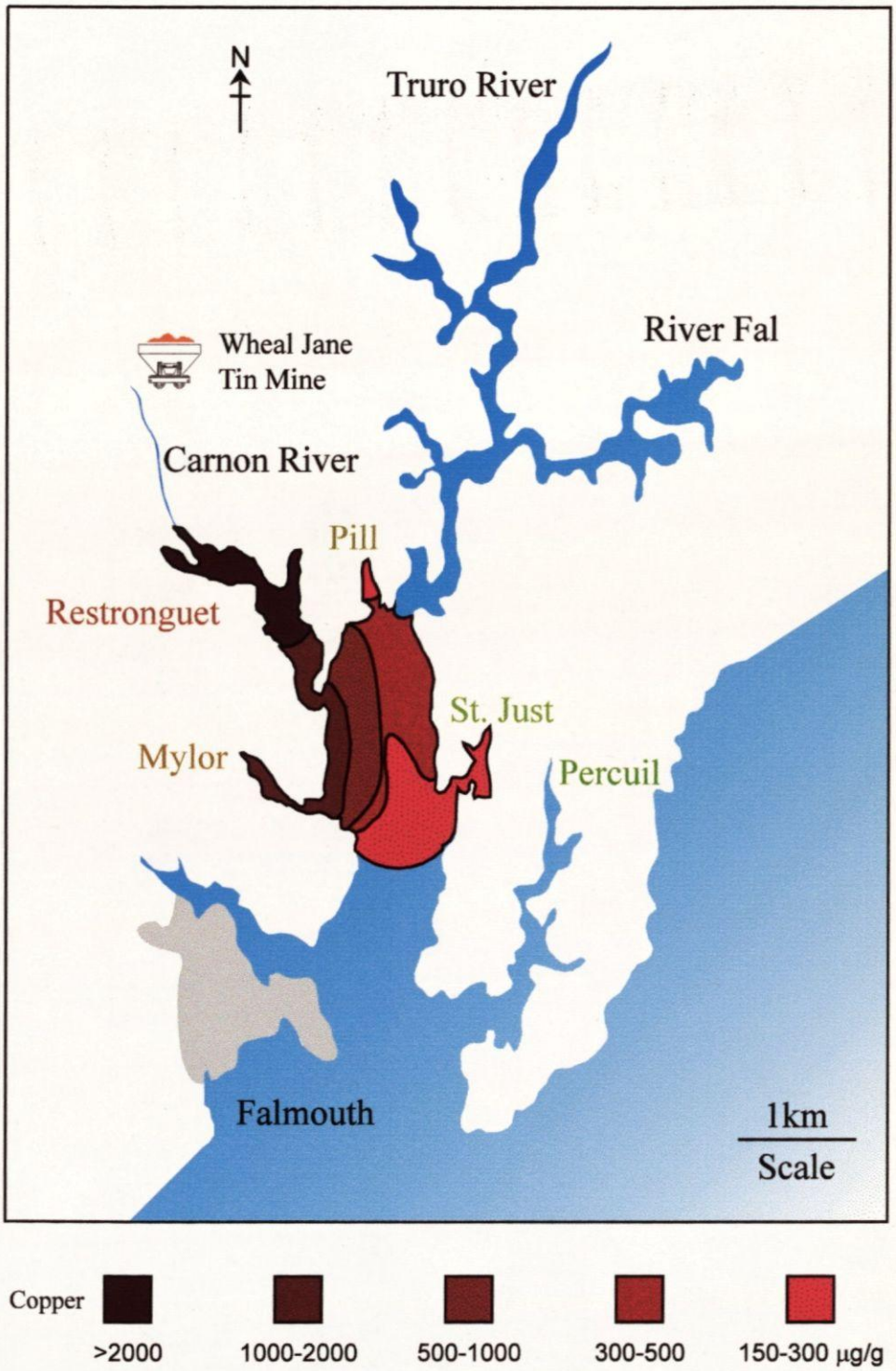

Figure 2.1 Fal Estuary Sediment copper concentrations, location of sample creeks and Wheal Jane tin mine (Data Source :- Bryan & Gibbs 1983).

The geology of the immediate area is shale and sandstone, and the surrounding countryside is low hills and wooded valleys. The creeks are dry at low water except for a narrow central channel. The estuary is very sheltered and stretches inland for nearly thirteen kilometres. It is surrounded by some agricultural land, ancient oak woodland and relatively undeveloped land. There is a wide range of habitats from rocky shores to extensive mud flats, salt marsh and sand banks. Sub-tidally there are oyster (*Ostrea edulis*) beds in addition to the maerl, forests of the peacock worm (*Sabella pavonina*) on some intertidal mud flats and many important fish species such as sea bass (*Dicentrarchus labrax*), the Cornish sucker fish (*Lepadogaster lepadogaster*) as well as species of wrasse and pipe fish found in certain areas (typically the south east side). However, it lacks the faunal diversity present in its neighbouring estuary, the Helford River, which has remained free of mining activities. It has been a centre of human activity for many centuries with fishing and farming of oysters still occurring. Commercial traffic in pottery, china clay and tin which were important exports has declined. It is now an important area for recreational activities such as sailing, windsurfing and angling.

Mining history

This present study focuses on heavy metal pollution. The Fal Estuary is the most heavily metal contaminated estuary in the UK (Bryan & Gibbs 1983). The surrounding area has a long history of mining, from the recovery of alluvial tin in the Bronze age to a peak of production in the 19th century when around 1000 mines produced 50% of the worlds' supply of copper, tin and arsenic. Output rapidly declined thereafter, with the last tin mine (Wheal Jane, Figure 2.1) closing in 1991. However, its flow of run-off water, contaminated with heavy metals, did not cease with closure. A continual build-up of rainwater in the old mine shafts needs processing. These waters are collected in a settling pool before being released into the Carnon River which flows into the Fal Estuary via Restronguet Creek. However, water levels in the mine fluctuate and plugs in mine shafts deteriorate and these,

coupled with the cessation of pumping, resulted in a huge spill in early 1992. Unprocessed waters were released into the Fal Estuary and flooded out to sea, observable as a rust red slick on the sea surface (Figure 2.2). Sick birds were seen, and warnings against bathing and the human consumption of shellfish from the area were issued. Thus the area has a long-term chronic contamination problem compounded by this recent acute pollution event

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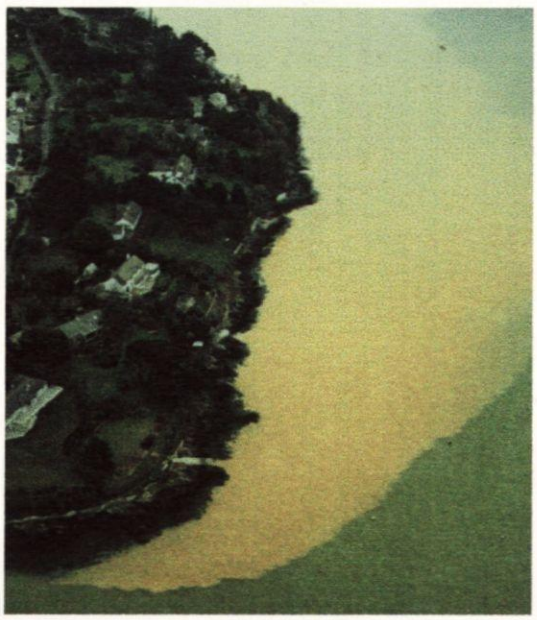


Figure 2.2 Plume of contaminated mine water advancing downstream towards Falmouth

Heavy metals in the Fal

The British estuaries most contaminated with heavy metals are located in the south-west and include those of Hayle (mainly copper pollution), Gannel (lead) and Looe (silver and lead). However the highest levels of copper, arsenic and zinc occur in Restronguet Creek, a branch of the Fal Estuary (Bryan & Gibbs 1983) (see Figure 2.1). Other important metals found include cadmium, iron and manganese. Via the Carnon River, Restronguet Creek drains parts of what was for several hundred years the most productive of the south-west mining districts, as well as an area of alluvial tin recovery stretching back to the Bronze Age. Since the cessation of this latter activity Restronguet Creek has been silting up. 19th

century photographs show ore ships alongside quays at Devoran at the head of Restronguet Creek which is almost inundated with sediments now. The metal pollution renders the water of the Carnon River acidic (pH 4).

Some metals are deposited whilst others remain in solution, resulting in varying proportions of metals. On entering Restronguet Creek the Carnon River water flows over the denser sea water and distinct stratification of the contrasting salinities can be observed in calm weather (Williams 1994). As the two mix, a rise in salinity and pH leads to the flocculation of iron as oxides (with other associated metals such as copper and arsenic), which are deposited leaving a reddish brown colour on the sediment surface. Metal concentrations in sediments decline only slightly downstream and remain fairly constant to a depth of 20 cm, apart from copper whose concentration at a depth of 60-65 cm is double that at the surface. This corresponds to approximately 100 years of sediment deposited since the time when copper mines reached the end of their greatest productivity. Sediment metal concentrations also decrease slightly with distance from the main channel.

Although the source of the contaminated waters lies in the Wheal Jane, a clear-cut gradient of heavy metals exists in the sediments (Bryan & Gibbs, 1983) throughout the estuary varying from, for copper, 2000 µg/g in Restronguet to less than 150 µg/g in Percuil Creek (Figure 2.1). Therefore sediments in otherwise similar creeks in different parts of the system have levels of heavy metals which differ by orders of magnitude (Bryan & Gibbs 1983, Somerfield *et al.* 1994a). Hence, this estuary has provided a range of pollution which has been used by various workers studying the effects of long-term metal pollution on marine organisms *e.g.* Bryan & Gibbs (1983), Bryan & Langston (1992), Grant *et al.*(1989), Somerfield *et al.* (1994a).

Zinc at the concentrations it occurs is toxic, but less so than copper which has the largest impact on flora and fauna (Harris & Somerfield 1995). In the presence of zinc, the toxicity of cadmium is greatly reduced (Bryan & Langston 1992). This heavy metal gradient is also found in shellfish tissue. Early studies in the Fal estimated metal concentrations in oysters with leucocytosis which causes the oyster tissue to become green due to build-up of copper (Boyce & Herdman 1898, Orton 1923). Williams (1994) who studied bio-accumulation in the polychaete, *Nephtys hombergi*, found that the gradient of metals existed in the worm, but at a concentration of copper reduced by a factor of ten compared to the findings of Bryan & Gibbs (1983) who studied the same species.

The Fal Estuary is an ideal experimental location for comparing monitoring methods as the creeks are uncomplicated by other pollutant factors (Somerfield *et al.* 1994a & b) and are standardised in terms of salinity and sediment grain size. Natural variables unrelated to the contamination that might influence the responses of the biological techniques were therefore minimised *e.g.* shore height, tidal height, sediment type, climatic and seasonal factors.

SAMPLING SITES

For reasons of comparison and continuity the choice of sites was based on those used by Perryman (1992) and Somerfield *et al.* (1994a & b) who undertook community analysis studies prior to and following the Wheal Jane tin mine spill of 1992. They had based their selection of sites upon the sediment copper data of Bryan & Gibbs (1983) to cover an expected gradient of heavy metal concentrations (Figure 2.1). The order of sediment metal concentrations of five chosen creeks ranged from highest values for copper near the tin mine at the head of Restonguet Creek ,which is the highest in the UK (Bryan & Langston

Within these selected creeks five replicate sites were chosen, with an additional two in Restronguet (Figure 2.4) representing similar sediment type and tidal height (the central part of the intertidal mudflats). These sites were used for the community studies of Chapter 3, the population studies of Chapter 4, and the environmental variable and bacteriological analyses of this chapter. The middle site in each creek was chosen for the position of deployed *Mytilus edulis* and hence the location for the techniques of scope for growth, larval survival studies and cellular damage assays of Chapters 5, 6 and 7.

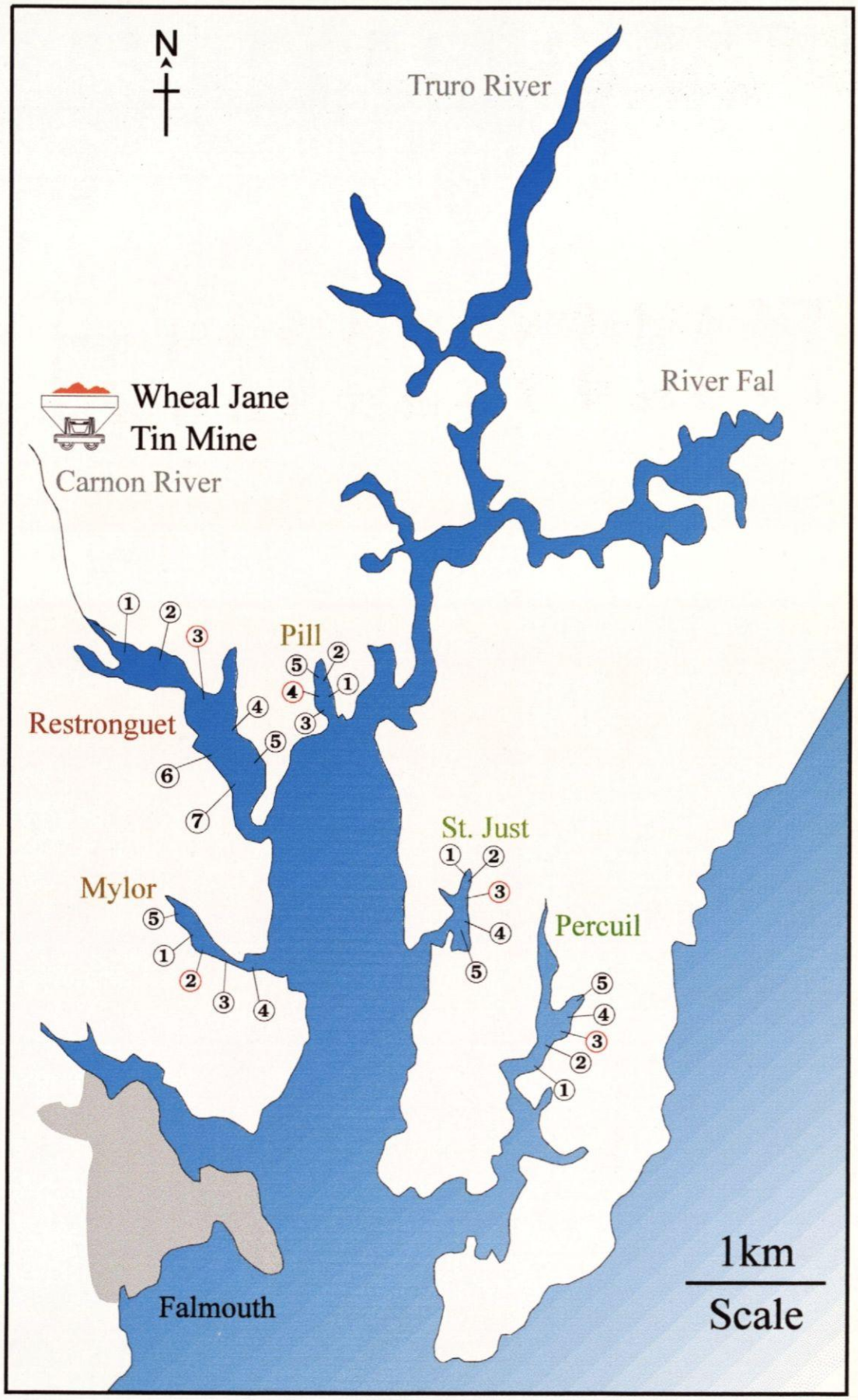


Figure 2.4. Location of sample sites in the Fal Estuary
(◯ indicates location of mussel cages)

Mussels (*Mytilus edulis*)

Mytilus edulis is absent from the Fal Estuary creeks under study, and occurs only in limited numbers in isolated locations near the central estuary channel of Carrick Roads. It was thus necessary to transplant mussels from a known clean site. Five hundred mussels were collected from Bull Hill in the Exe Estuary (Figure 2.5) in May 1994. They ranged in size from 40 to 50 mm, and were removed by carefully cutting their byssus threads, then transferred to the Fal Estuary in cool boxes. They were left in cages (45 x 35 x 10 cm: mesh size 2.5 cm) (Figure 2.6) attached to wooden stakes driven into the sediment. One cage containing 100 mussels was placed in the middle site of each creek, higher up the shore than the community samples, on hard substrate (gravelly sand) to enable firm attachment of the cage and avoid excessive influence of fine sediment.

Figure 2.5 Bull Hill mussel beds in the Exe Estuary

Figure 2.6 Samples collected from mussel cages in Percuil Creek

Why use mussels as experimental organisms ?

Mussels have been the most popular organisms for monitoring chemical contamination and pollution stress for various reasons (after Widdows & Salkeld 1992b).

Mussels are:

- Sedentary and thus unable to avoid contamination in their surroundings (although they can temporarily isolate themselves from high contamination exposure by closing their valves);
- Abundant and widely distributed and thus available regionally and globally for monitoring;
- Unaffected by handling stress and thus amenable to transplantation to sites lacking indigenous mussel populations;
- highly sensitive and responsive to sublethal levels of pollutants, enabling stress effects to be detected before mortalities occur;
- tolerant of a wide range of environmental conditions;

- bioaccumulators of chemical contaminants by factors of 10 to 100,000 thus facilitating analytical detection of contamination;
- suspension feeders that effectively 'sample' large volumes of water in the region of 4 litres per hour;
- commercially important shellfish.

MATERIALS AND METHODS

Sediment metal analysis

Sampling

Sediment samples were taken in November 1992 and November 1993 at each location (*i.e.* five sites per creek and seven in Restronguet) for comparison with those collected in November 1991 (by Perryman 1992 and Somerfield *et al.* 1994a and b). Changes in trace metal content due to contamination are important as is prevention of contamination during sampling and storage *e.g.* from storage vessels. Surface sediment scrapes for heavy metal analysis were collected in acid washed containers (placed in acid bath of 10% hydrochloric acid (HCl) for 24 hours and rinsed with Milli-Q water and drip-dried; polyethylene, rather than glass, to prevent adsorption onto the walls of the sample container). They were transported to the laboratory and frozen at -20^{o}C within 8 hours, to prevent any chemical transformations in the sediment occurring before subsequent analysis.

Metal extraction by microwave acid digestion

The analytical technique required liquid samples and thus the sediment samples had to be pre-treated with acid to bring the samples into solution. This involves destruction of organic matter by a wet extraction method using microwave heating, which speeds up digestion as compared to heating on a hot plate, and subsequent quantitative recovery of the metals.

For extraction of metal, sediment samples were wet sieved (with Milli-Q water) through acid washed 63 μm nylon sieves into acid washed containers. Use of the fine fraction for analysis minimises the variation caused by sediment particle size. These samples were then freeze dried. One gram of each sample was added to a large digestion tube (120 ml) then digested in concentrated nitric acid (HNO_3), and the residues dissolved in M HCl. A blank was prepared for each run using the HNO_3 and HCl. The tubes were heated in a microwave oven (MDS-2000) at 100% power, 80 PSI for 5 minutes, then at 100% power, 160 PSI for 20 minutes. After this procedure the samples were centrifuged for separation of the extract, decanted off and made up to 25 ml using concentrated HNO_3 and stored in acid washed bottles at $4^{o}C$ until metal analysis was completed.

Metal determination

Metal concentrations were determined by flame atomic absorption using a Varian AA20 atomic absorption spectrometer with auto sampler. Background correction was used for all elements other than copper, zinc and iron. An air/acetylene flame was used for all metals except for chromium for which a nitrous oxide/acetylene flame was used. A set of standard solutions were prepared for calibration, from which the concentrations of elements in each sample solution were determined. Metals analysed each year were copper, zinc, manganese and iron. Additional metals (cadmium, cobalt, chromium, nickel and lead) were determined in 1993 (and in 1991 by Somerfield *et al.* 1994a and b).

Mussel (*Mytilus edulis*) metal analysis

Mussels were collected from the cages in each of the five creeks as well as from their site of origin in the Exe and transferred to the laboratory in cool boxes. They were left in clean seawater overnight for depuration of sediment. The flesh was dissected from the shells and the stomach removed in case of sediment metal contamination as it would take 36 hours for elimination of most contaminated gut contents (Robinson *et al.* 1993) The remains of each animal were freeze dried.

Metal extraction by microwave acid digestion

For determination of metal concentrations in mussel tissue 0.5 g of each sample was added to a large digestion tube then digested in 5 ml concentrated HNO_3 (aristar nitric acid) and 2 ml hydrogen peroxide (H_2O_3 - analar grade). Blanks were prepared for each run using the HNO_3 and H_2O_3. The tubes were left overnight and then microwaved at 100 % power, 80 PSI for 10 minutes. The samples were made up to 25 ml with HNO_3 and stored in acid washed bottles at 4°C until metal analysis was completed. Reference material (DORM-1 dogfish tissue) also underwent a parallel procedure.

Metal concentrations were determined by flame atomic absorption using a Varian AA20 atomic absorption spectrometer with auto sampler. Background correction was used for all elements other than copper, zinc and iron. An air/acetylene flame was used for all metals. Metals analysed were copper, zinc, manganese and iron.

Environmental variables

Sediment grain size & organic matter, pore water salinity and pH.

Sediment samples were taken, at each date above, from each location in 0.5 litre containers, transported to the laboratory and frozen at -20°C pending subsequent analysis for percentage silt/clay and percentage organic matter. Pore water salinity and pH were tested in 1993 *in situ* in the field by refractometer and pH meter.

The percentage silt/clay in sediment samples was estimated by wet sieving through a 63μm sieve, drying at 95°C and weighing. The percentage organic matter was determined by loss of weight on ignition at 600°C after removal of carbonates by treatment with 8% H_2SO_3 (sulphurous acid).

Bacteriology of estuary water

Bacterial content for each creek was determined in July 1995. Water was collected at high tide in acid-washed containers from the same locations described above at high water. Analysis was by membrane filtration methods followed from 'Methods for examination of waters and associated materials' Report No. 71 (1982):

Faecal streptococci.

Test volumes of water (100 ml) were filtered through membranes by vacuum pump. Each membrane was incubated on an agar medium (the Slanetz and Bartley glucose azide medium) at 37°C for 48 hours and then at 45°C for a further 48 hours. All red, maroon or pink colonies were counted as presumptive faecal streptococci.

Escherichia coli.

Test volumes (100 ml) of the water samples were filtered through a membrane of cellulose esters. The filters were then placed face-upwards on an absorbent pad saturated with broth. White colonies of *E. coli* were counted after incubation.

RESULTS

Sediment metal analysis

Sediment metal concentrations are shown in Table 2.1 and illustrated by Figure 2.7. It is apparent that the gradient first noted by Bryan & Gibbs (1983), on which the sampling design was based, still exists despite the fact that the Wheal Jane tin mine has ceased operating. Concentrations of heavy metals were lowest in Percuil Creek and approximately doubled-up through St. Just, Pill and Mylor creeks to highest levels in Restronguet Creek. One-way ANOVA confirms that the differences between the creeks were significant ($p < 5\%$) (see Appendix I for complete data set).

Sites	Date	Copper (µg/g)	Zinc (µg/g)	Manganese (µg/g)	Iron (mg/g)
Restrong.	**1991**	2500 ± 580	3800 ± 800	540 ± 44	56 ± 8
	1992	2200 ± 710	5900 ± 900	420 ± 75	62 ± 8
	1993	2700 ± 470	4700 ± 990	425 ± 54	77 ± 17
Mylor	**1991**	1300 ±62	1400 ± 59	400 ± 15	41 ± 1.4
	1992	1100 ±196	1700 ±330	380 ±77	42 ± 7.6
	1993	1200 ± 92	1500 ± 150	340 ± 72	41 ± 6
Pill	**1991**	700 ± 19	1000 ± 69	280 ±13	34 ± 1
	1992	690 ± 99	900 ±130	230 ± 51	35 ± 7
	1993	520 ± 45	800 ± 90	230 ± 35	33 ± 11
St. Just	**1991**	330 ±33	620 ± 33	250 ± 10	29 ± 2
	1992	390 ± 100	610 ± 110	240 ± 72	27 ± 5
	1993	370 ± 36	590 ± 64	240 ± 80	35 ± 5
Percuil	**1991**	170 ± 34	302±62	220 ± 39	32 ± 7
	1992	191 + 16	330 ± 73	230 ± 78	38 ± 5
	1993	160 ± 35	290 ± 48	220 ± 82	39 ± 8

Table 2.1. Heavy metal concentrations(mean ± SD) in sediments from five creeks in the Fal Estuary (n = 5, except for Restronguet where n = 7), November 1993, 1992 and 1991 (data for 1991 from Perryman 1992).

Mussel (*Mytilus edulis*) metal analyses

Metal concentrations, *i.e.* bioavailable metal levels, of mussel tissue are shown in Table 2.2, Figure 2.8. The values show the gradient of metal contamination to be associated with levels in the creek sediments, and to differ significantly between creeks ($p < 5\%$).

Site	Copper	Zinc	Manganese	Iron
Restronguet	27 ± 8.1	350 ± 56	3.8 ± 0.3	240 ± 3.4
Mylor	6.6 ± 2.6	220 ± 20	3.0 ± .0.2	100 ± 12
Pill	6.7 ± 3.6	97 ± 50	2.3 ± .03	96 ± 15
St. Just	3.3 ± 2.0	61 ± 20	2.8 ± 0.6	81 ± 9.3
Percuil	2.2 ± 0.8	53 ± 7	1.9 ± 0.3	82 ± 22
Exe	2.4 ± 0.7	52 ± 12	2.4 ± 0.5	78 ± 15

Table 2.2 Heavy metal concentrations (mean ± S.D) in mussel flesh (μ/g). (n = 5, except for Restronguet where n = 7).

Environmental variables

Sediment grain size and organic matter.

It was important to choose sites which had as few variable factors as possible apart from their heavy metal content. The variables (Table 2.3, Figure 2.9) do not differ significantly between creeks, although St. Just and Mylor had slightly higher organic carbon content due to leaf litter from the surrounding overhanging woodland and Restronguet had slightly higher percentage fines.

Site	1991		1992		1993	
	% o.m.	% fines	% o.m.	% fines	% o.m.	% fines
Restronguet	6.2 ± 1.4	77 ± 15	8.4 ± 0.4	85 ± 2.3	7.6 ± 1.1	72 ± 25
Mylor	8.4 ± 0.3	96 ± 2.5	10 ± 0.5	94 ± 3.1	10 ± 0.5	94 ± 3.2
Pill	9.1 ± 0.3	94.2 ± 2.5	8.8 ± 0.4	94 ± 3.6	9.3 ± 0.4	94 ± 3.3
St. Just	13 ± 4.3	93.0 ± 2.8	14 ± 2.6	94 ± 2.3	17 ± 1.0	94 ± 3.8
Percuil	8.5 ± 1.1	94.8 ± 2.6	9.4 ± 0.8	96 ± 2.6	8.4 ± 1.0	94 ± 2.2

Table 2.3 Percentage silt/clay and percentage organic matter of sediments from five creeks in the Fal Estuary sampled in

November 1993, 1992 and 1991
(Mean ± SD, n = 5 and n =7 for Restronguet).

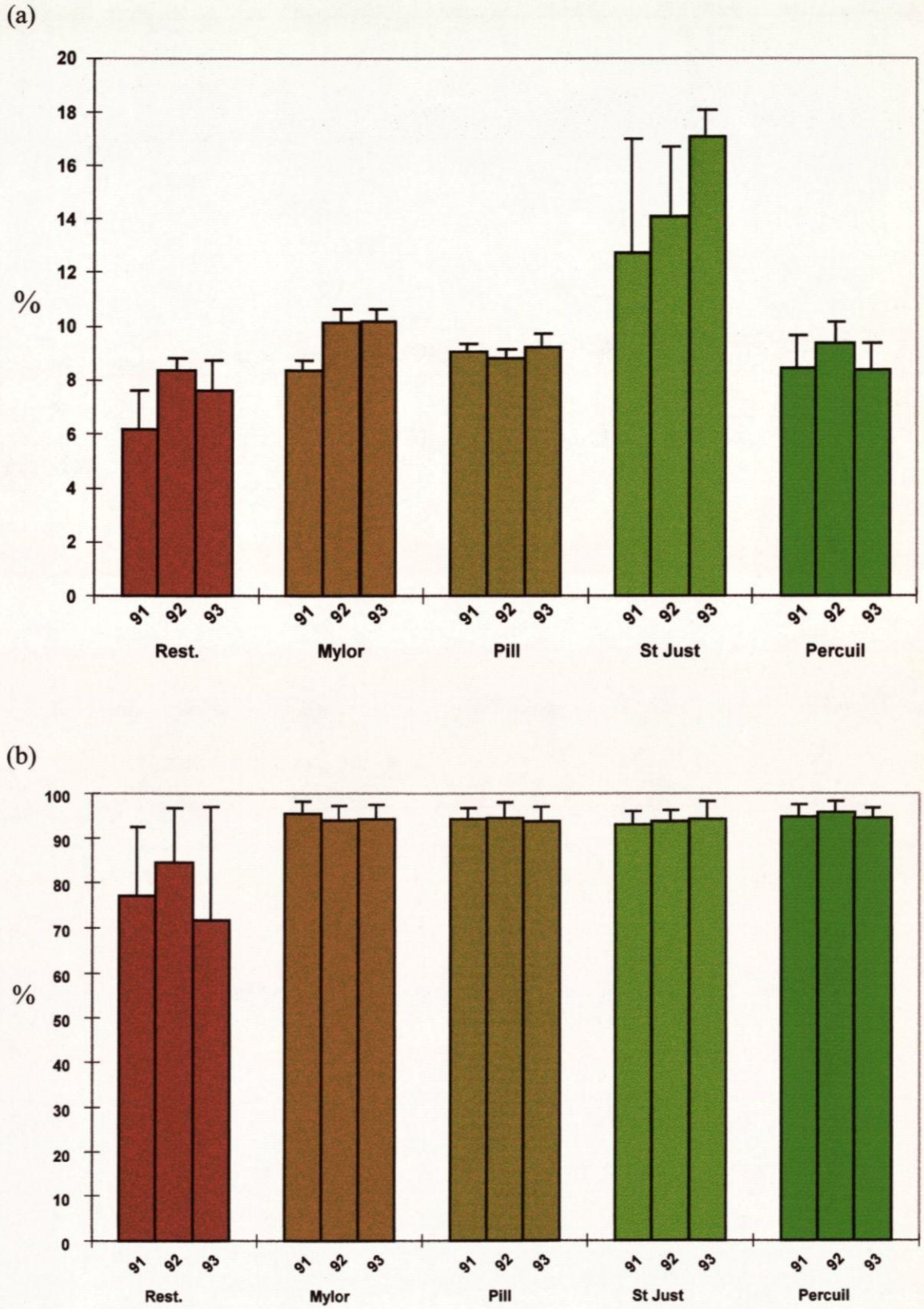


Figure 2.9 Sediment percentage organic matter and fines 1991 - 1993

(a) % organic matter (b) % fines
(Error bars indicate standard deviation)

Pore water salinity and pH

Pore water salinity and pH values are presented in Table 2.4 and Figure 2.10. Salinity values supported the observation in the introduction that the estuary is essentially fully saline. However, there were sites such as Pill 1 and St. Just 4 that exhibited lower salinity on the day of testing. This may have been due to intermittent streams that enter the sides of these two creeks.

Site	Salinity	pH
Restronguet	30 ± 1.5	7.0 ± 0.1
Mylor	28 ± 0.5	6.6 ± .03
Pill	28 ± 5.9	6.7 ± .01
St. Just	26 ± 8.2	6.5 ± .03
Percuil	31 ± 1.5	6.5 ± 0.3

Table 2.4 Pore water, salinity and pH of sites in five creeks in the Fal Estuary November 1993, (n = 5 and n = 7 for Restronguet).

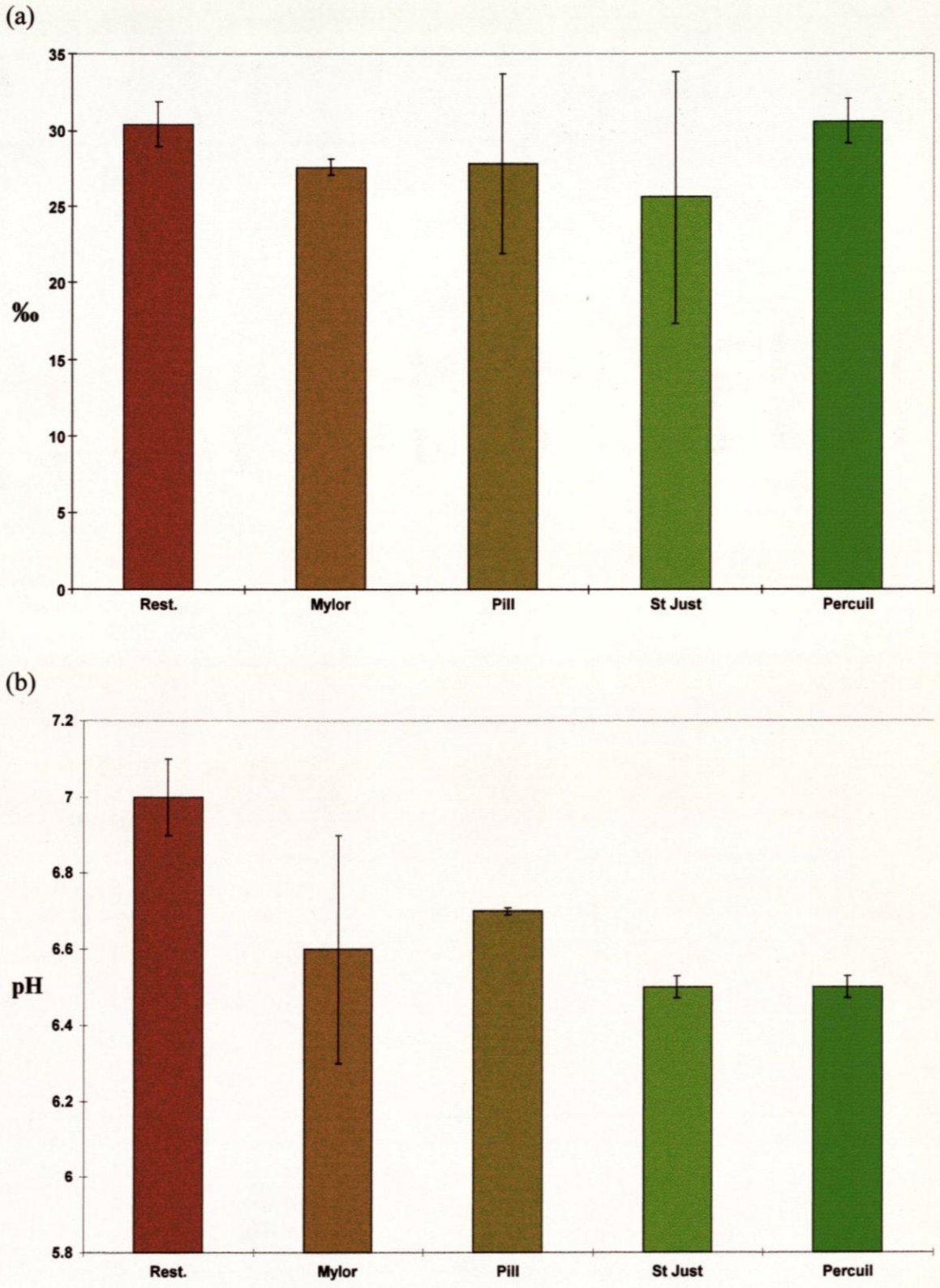
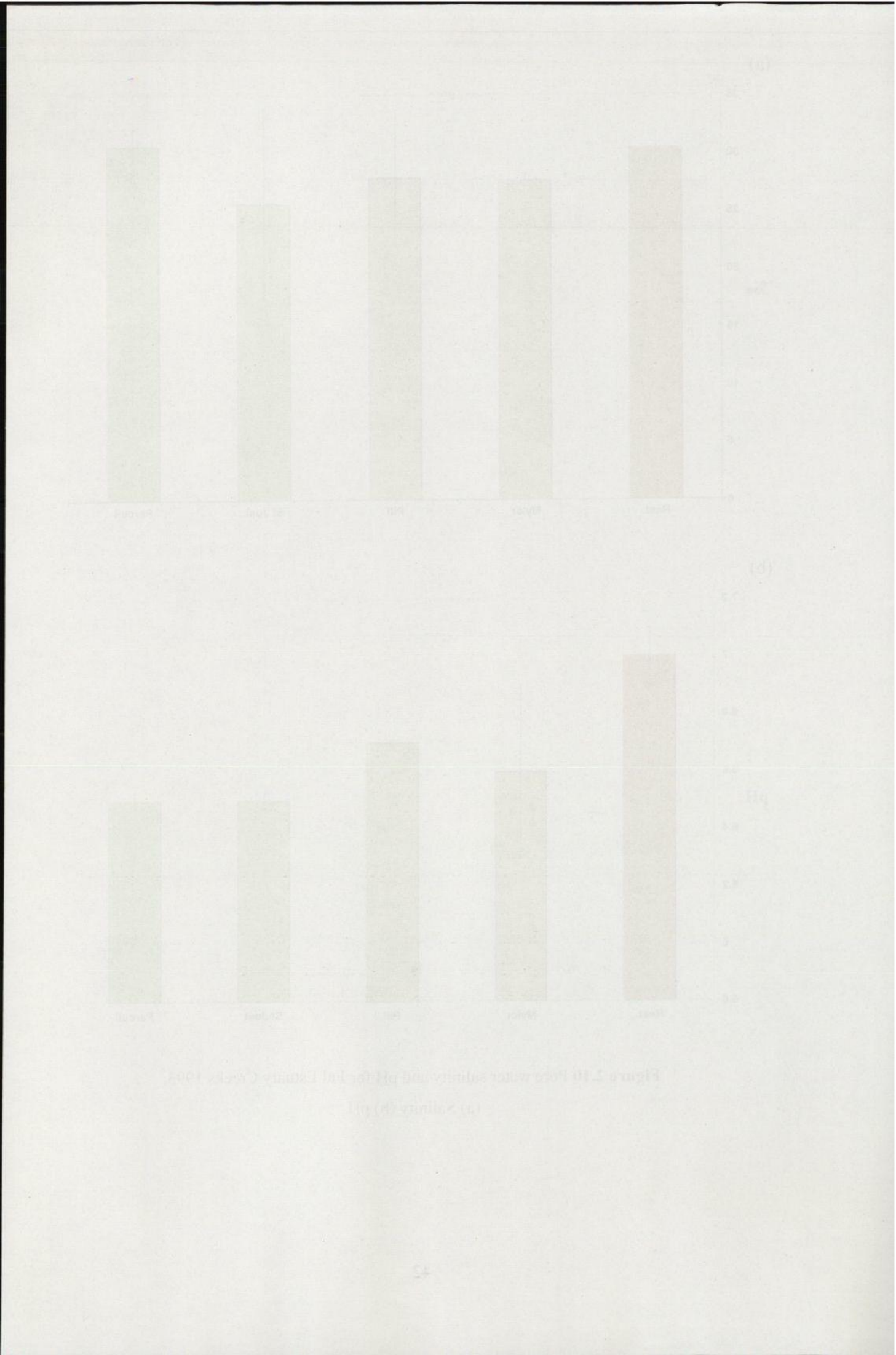


Figure 2.10 Pore water salinity and pH for Fal Estuary Creeks 1993
(a) Salinity (b) pH



Bacteriology of estuary water

Results for of the analyses *Escherichia coli* and faecal streptococci are presented in Table 2.5. All levels are well below the E.C. recommended Bathing Beach Limits (2000/100 ml for *E. coli*) and hence are not an obvious source of organic pollution.

Creek	Site	*E. coli* per 100 ml	Faecal strep. per 100 ml
Restronguet	1 Top	28	0
	3 Middle	46	2
	5 Bottom	4	1
Mylor	5 Top	20	107
	2 Middle	32	26
	4 Bottom	260	4
Pill	5 Top	120	17
	4 Middle	14	8
	3 Bottom	252	26
St. Just	1 Top	4	1
	3 Middle	0	0
	5 Bottom	26	2
Percuil	5 Top	24	0
	3 Middle	6	2
	1 Bottom	4	2

Table 2.5 Results of Fal Estuary water sample bacteriology tests (20/7/95) for number of colonies of *E. coli* and faecal streptococci from three sites per creek (top, middle and bottom).

CONCLUSIONS

The heavy metal gradient in the sediment persists. Mussels transplanted to the creeks accumulate selected metals in direct relation to the sediment levels. Other environmental variables do not differ significantly between the creeks which means that the required experimental environment for the comparison of techniques existed.

CHAPTER 3

Community Structure Analysis

INTRODUCTION

Background

The analysis of changes in benthic community structure have become the popular and routine way to detect and monitor the biological effects of marine pollution. Methods employing lower levels of biological organisation reflect the condition of the organisms only at the time of sampling, whereas the structure of an assemblage of organisms, *i.e.* the community, reflects the integrated conditions over a period of time. By its very nature it is the community that is of direct concern, and predicting consequences from lower level signals is not practical at present. Monitoring at the level of ecosystem is unmanageable and so in practical terms communities are the most apposite (Warwick 1993).

However, benthic community monitoring is not without criticism. It is highly labour intensive, both in terms of sampling effort in the field and in the analysis of samples in the laboratory, and correspondingly expensive. Also it cannot readily be translated to regions of the world where the fauna is poorly known and taxonomic expertise lacking. It said that when anthropogenic changes in the community structure are apparent damage has gone too far and it is too late to protect the environment (Depledge & Hopkin 1994). Another

criticism is that it is difficult to separate changes induced by man's activities from those resulting from natural environmental variables such as water depth and sediment type, which can confound sampling design and data interpretation. Also, it is argued that methods of interpreting community data are intuitive rather than objective relying on experience of the experimenter and this is not conducive to standardisation or routine monitoring operations on a wider scale.

More recently, these perceived problems have been broached and discussed at workshops organised by the Group of Experts on the Effects of Pollutants (GEEP). They produced no evidence to support claims of the early-warning or higher sensitivity of low biological level assays. Deaths of organisms are not necessarily required for a community response. Multivariate methods of data analysis are sensitive in detecting subtle responses of species, to pollution (Warwick 1993). The need for taxonomic expertise has been rectified by realising that decreasing taxonomic levels in analysis does not loose significant amounts of information, and this factor increases their cost effectiveness and facilitates world-wide application. Protocols for analysing communities have been established (Clarke 1993, Underwood 1993).

Monitoring the effects of pollutants at the community level has a large literature describing how patterns of species abundance respond to pollution. Heip *et al.* (1988) analysed the community attributes of the benthic meiofauna of Frierfjord / Langesundfjord for the GEEP workshop; Gee *et al.* (1992) have analysed the meiofauna community response to environmental pollution associated with the site of a former oil rig in the German Bight; Austen *et al.* (1989) studied the community structure along a pollution gradient in southern Portugal; Gobin (1988) looked at the polychaete macrofauna near a large industrial

complex in Trinidad; Olsgard & Hasle (1993) considered impact of mining waste; and Pearson & Rosenberg (1978), organic enrichment.

More relevant to this present study, Somerfield *et al.* (1994) and Perryman (1992) looked at the benthic community structure of the Fal system before and after the infamous Wheal Jane tin mine spill of 1992. Contrary to contemporary reports that it was a major catastrophe for the local marine environment, analyses showed no changes in the sediment heavy metal content, and only minor changes in the meio- and macro-benthic communities which could be attributed to natural seasonal fluctuations. They considered that of the three components of the fauna studied (nematodes, copepods, macrofauna), nematodes gave the best relationship with the heavy metal gradient because nematode community structure changed in an ordered fashion with increasing metal concentration established over time. They found that the communities of copepods and macrofauna responded in a similar way to one another in that those in Restronguet differ from the rest, but that the communities in the other four creeks are not ordered according to the gradient, and concluded that other factors influence these communities. Subsequently, Milward & Grant (1995), using toxicity tests on the whole nematode community from creeks of the Fal system, showed spatially varying resistance to copper. This is as a result of an increase in the abundance of copper-resistant species, the evolution of enhanced copper tolerance in some species and the exclusion of more sensitive species. They found that Restronguet was distinct from Percuil Creek and inferred that this is a product of the copper contamination and so suggested that pollution-induced community tolerance may be used as a tool to evaluate chronic pollutant impact on marine benthos.

Unstressed, pollutant free communities which are at a late successional stage are characterised by a range of distinct species belonging to many phyla, whereas stressed and polluted situations result in communities with a low species diversity (Warwick & Clarke 1995). Of course, these are extremes and there are intermediate situations. However, the diversity may vary at different levels of taxonomic classification. Two sites with the same value of species diversity index may either have species which are closely related taxonomically, *e.g.* a variety of polychaetes, or have more differing families and phyla, *e.g.* a range of bivalves, polychaetes and decapods. Individual species vary in their tolerance to pollution. Sensitive species will decline in numbers, tolerant ones will remain unaffected, or may even benefit. Such changes are the subject of benthic pollution monitoring.

'Indicator' organisms can be characteristic of certain conditions. For instance, *Capitella capitata* is a pollution indicator and plays a similar indicator role in marine waters to the oligochaete *Tubifex* in freshwater (Hart 1979). The use of such indicators is based on the understanding that any environment will have balanced biological conditions and contain a high diversity of life with no one species dominating. If a pollutant is discharged into an area the most sensitive species will be killed and the more tolerant species may lose their predators and so increase numerically. Therefore, a knowledge of the species present and their characteristics is important in evaluating the effect of pollution (see Appendix 2).

Taxonomic levels and community studies

The species composition of benthic assemblages in relation to pollution gradients has been described by applying various hierarchical levels of taxonomic aggregation to species data with no substantial loss of information at the higher levels (Warwick 1988, Heip *et al.* 1988). Data from a variety of sites from the NE Atlantic have been aggregated to phylum level and abundance and biomass data merged to generate a 'production matrix'. A multi-dimensional scaling (MDS) ordination of these data, combined in a single *meta-analysis*, produced a configuration used as a training data-set against which it was proposed that the pollution status of communities from new studies could be assessed (Warwick & Clarke 1993).

Warwick (1988) suggested that anthropogenic environmental variables may induce community responses at a higher taxonomic level than natural environmental variables, which influence the fauna by replacement of species, and that this differential response could be potentially useful in separating pollution effects from natural environmental variables. He proposed that this could lead to the solution to one of the major problems of interpretation of benthic community data in respect to pollution effects. Somerfield and Clarke (1995) took this further introducing the concept of a 'second stage' MDS, in which rank correlations between pairs of similarity matrices themselves become the elements of a second similarity matrix, an ordination of which gives the summary of the conclusions. They found that both sub-littoral and intertidal macrofauna communities are robust to family level, and the latter to phyla with only minimal effect on consequent analysis. They concluded that whichever component of the benthos is examined, and whatever taxonomic level is analysed, interpretable results are possible, especially if the pattern of community change is marked. In this present study such an approach has been tested as the typically

low diversity of an estuary is thought unsuitable (Kendall pers. comm.) for analysis at higher taxonomic levels.

Benthic infauna

Studies of infaunal benthic communities have been used because these communities are relatively immobile and persistent when compared to pelagos or epibenthos. Infauna respond to localised conditions and so the community structure at any one site can be viewed as an integrated response to environmental conditions. Macrofauna have been the component of the benthos traditionally examined in pollution monitoring studies, although recently, meiofauna have been used (Somerfield *et al.* 1994b) in preference. Meiofauna have several advantages, *e.g.* they have a potentially shorter response time due to shorter generation times, the fauna are smaller and in higher density and therefore smaller and more convenient samples are needed. Also they are more sensitive to anthropogenic disturbance (Heip *et al.* 1988 and Warwick 1993). However, the advantages of using macrofauna of the soft-bottom benthos are (Warwick 1993):

1) They are relatively non-mobile and are therefore useful for studying the local effects of pollutants.

2) Their taxonomy is relatively easy.

3) Their response to perturbation at high taxonomic levels has been better tested than other components of the biota.

4) Quantitative sampling is moderately easy.

5) There is an extensive research literature available on macro-benthic communities against which comparisons can be made.

But there are also disadvantages:

1) Relatively large volumes of sediment samples are required.

2) Transporting large volumes of sediment to the laboratory for processing is impractical so sieving is unavoidably done in the field which is time consuming and often inconvenient.

3) The potential response time of the macrofauna to a pollutant event and its subsequent recovery is slow (due to long generation times) and thus the full establishment of a community characterising the new environmental conditions may take several years.

4) The baseline with which anthropogenic effects are compared is inherently unstable as there are erratic natural temporal changes in community structure due to *e.g.* the inconsistent recruitment and settlement of macrofauna pelagic larvae with resultant dramatic changes in the abundance of individual species occurring from year to year.

Community data analysis

The computer software package PRIMER (Plymouth Routines in Multivariate Ecological Research) aids analysis and interpretation of community structure data. The methods reduce complex species and site data to a form that is easily interpreted visually. It is convenient to categorise the analyses into four main stages.

Representing communities

Community data can be represented and described visually, using graphs, which reduces their complexity but does not explain or test the data. This is rather a 'pure' description with the emphasis on reducing the complexity of the multivariate information to obtain a low-dimensional picture of how the biological samples interrelate.

Discriminating sites

Sites or conditions can be discriminated and identified on the basis of biotic composition. The hypothesis tests whether there are statistically significant community differences between groups of samples, for instance demonstrating differences between control and suspected impacted sites, or establishing before / after impact differences at a single site.

Determining levels of stress

Disturbance levels can be determined by biological measures which are indicative of perturbed conditions, and can ascribe directionality to observed change.

Associating biotic data to environmental variables

With the picture given by the biological data, physical and chemical data can be examined for their own structure and related to the biotic pattern. However, relationships cannot be established as 'causal' in this way, and can only be determined by manipulative experiments.

Aims

The purpose was to investigate the composition of the soft-bottom intertidal fauna in selected creeks of the Fal Estuary over a three year period, to describe the physical and chemical composition of the bottom sediment, to describe the changes in the composition of the infauna both spatially and temporally, and to establish possible reasons for and relationships between the observations using univariate diversity indices and multivariate analysis.

MATERIALS AND METHODS

Sampling

Sampling sites have been described in Chapter 2. Somerfield *et al.* (1994a & b) and Perryman (1992) sampled in November 1991, before the imminent spill from the Wheal Jane tin mine and in March 1992, after the release of contaminated water. The same locations were sampled in November 1992 and 1993 for this study. Hence three continuous years of data were available for benthic macrofaunal community structure studies. Figure 2.4 (Chapter 2) shows the location of the creeks and sampling sites of the Fal Estuary. At each location, and on each of the survey dates, cores of sediment were taken, 20 cm deep with a diameter of 20 cm, for subsequent identification of macrofauna (See Figure 3.1). Thus from each creek there were five replicate cores (and seven for Restronguet).

Figure 3.1 Sampling benthic macrofauna with sediment corer in Percuil Creek

Sample processing and laboratory analysis

Macrofaunal samples were sieved through a 500 μm sieve to reduce the samples to a manageable size. The residue was fixed for preservation in 4% formalin, buffered because formalin tends to become acid causing damage to bivalve shells. It was then stained with 5% Rose Bengal, a proteinaceous dye, to facilitate accuracy of sorting and stored in 80% alcohol pending later identification. Before sorting, the samples were thoroughly washed

with water to limit exposure to formalin, a known carcinogen. Some samples with an exceptional abundance had fauna elutriated from the alcohol to ease the sorting workload. This involved passing a continuous stream of water through the sample in a container with an overflow to a sieve. The flow rate was adjusted so that the water agitated the sample carrying the small animals over without the majority of the debris. These were then sorted to species by hand under a low power binocular microscope, identified and counted. After sorting the animals were transferred to 80% alcohol for storage.

Analyses of community structure data

Two main approaches were used to extract workable representations and summaries of the biological data :

Univariate Analysis

Univariate diversity indices break down a full set of species counts for a sample to generate a single coefficient. Species do not retain their identity in the calculations. These are relative measures, for use in comparisons within a study rather than having an absolute global value application (Clarke 1993). The following indices were determined:

Total numbers of individuals (A) and of species (S).

Index of diversity by Shannon-Wiener, H':

$$H' = -\Sigma_i \, p_i(\log p_i)$$

where p_i is the proportion of the total count arising from the ith species.

Equitability, the extent to which the community counts are dominated by a small number of species expressed by *Pielous's evenness index, J'*:

$$J' = H'(observed) / H'max$$

where $H'max$ is the maximum possible diversity which would be achieved if all species were equally abundant.

Species richness, an estimate of the numbers of different species for a fixed number of individuals expressed by *Margalef's index, d*:

$$d = (S - 1) / \log N$$

where N = total number of individuals.

Each was calculated using the program DIVERSE in PRIMER. The latter index is the most commonly employed measure of diversity, but is biased towards species richness. The significance of differences of macrofauna diversity was tested using one-way ANOVA.

Multivariate Analysis

Multivariate statistical techniques discriminate between samples on the basis of their faunistic attributes taking into account not only the distribution of individuals among species but also their identity, and hence are more sensitive than univariate measures in discriminating between sites (Clarke & Warwick 1994). This method involved a series of analyses and approaches using PRIMER:

Dendrograms and MDS (Multi-dimensional scaling) plots.

The recorded observations from the twenty seven sites were subjected to computation of triangular matrices of similarities between all pairs of samples. Similarity coefficients facilitate a classification or clustering of samples into groups which are mutually similar, or an ordination (MDS) plot in which samples are 'mapped' in such a way that distances between pairs of samples reflect the relative dissimilarity of species composition. The similarity of every pair of sites was computed using (a) the Bray-Curtis index on square and double square root transformed species abundance data and (b) the Euclidean distance similarity coefficient for the environmental data. Clustering of stations into distinct groups was by an hierarchical, agglomerative method using group average sorting, the results of which are displayed in a dendrogram in which the ordering along the axis is not particular.

Transformations (square root and double square root) were used to reduce the effect of very dominant species thus allowing the importance of less abundant species to be recognised in the tests; for example, within one individual sample, oligochaetes tend to be present in hundreds and thousands whereas bivalves and crustacea may be present in single numbers. Ordination was by non-metric multidimensional scaling (MDS) and the significance of differences, both spatial and temporal, was established using the program ANOSIM (Kruskal & Wish 1978, Clarke & Green 1988). The program SIMPER (Clarke 1993) was used to establish the contribution of individual species to the dissimilarities between both creeks and years. An Index of Dispersion (using the program MVDISP) was calculated to determine the variability within samples, which can be a symptom of stress (Warwick & Clarke 1993)

Analysis at varying levels of taxonomic organisation.

The program AGGREG was used to analyse the species data at different levels of taxonomic distinction. Macrofauna abundances were aggregated to the level of family, class and phylum. A Spearman rank correlation (*P*) was computed between the corresponding elements of each pair of matrices, and the significance of the correlation determined by a permutation procedure using the PRIMER program RELATE (Clarke & Warwick 1994). Again ordination was by non-metric multidimensional scaling (MDS) as described above.

Analyses of environmental variables

Environmental data comprise whole numbers as compared to community data which typically see numerous zero values in a matrix and therefore ordination is by a Principal Components Analysis (PCA). The significance of any differences was tested by ANOSIM (Clarke 1993) applied to the Euclidean distance matrix underlying the ordination and by two-way nested ANOVA. Log transformations were applied to the data to transform the distributions to normality.

Linking the faunistic data to the environmental data.

The program BIOENV (Clarke & Ainsworth 1993) was used to test the relationship between environmental variables and the community structure of the macrofauna. It shows how well faunistic and environmental ordinations match. BIOENV computes a rank correlation coefficient (weighted Spearman coefficient) of all the elements of the two triangular similarity / dissimilarity matrices underlying the ordinations of environmental variables and the biota. The combination of variables which best explain the community structure is that giving the highest rank correlation coefficient.

RESULTS

Environmental variables

Sediment metal concentrations for each of the three years have been presented in Table 2.3 (Chapter 2). Ordination of the 1993 metals data by Principal Components Analysis (PCA) (Figure 3.2) shows that all creeks are clearly separated from one another, following the approximate order of metal gradient. ANOSIM confirmed that each creek differs significantly ($p < 5\%$). Other environmental variables (Table 2.3, Chapter 2) are illustrated as PCA plots (Figure 3.3) and show that all sites, with the exception of R6 and R7, are similar in terms of natural environmental variables.

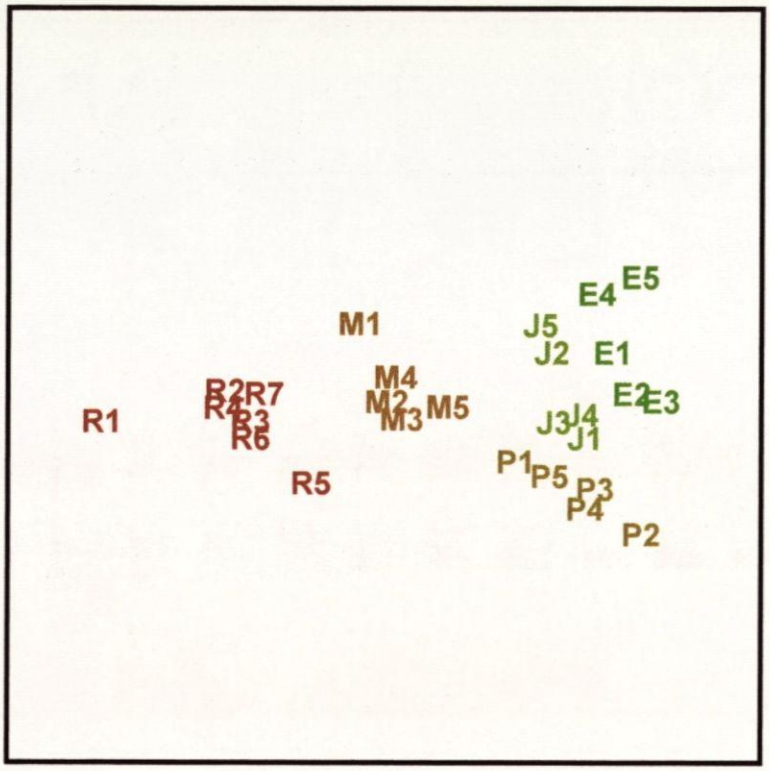


Figure 3.2 Ordination by correlation based PCA of 4th root transformed sediment metal data from Fal Estuary creeks 1993.

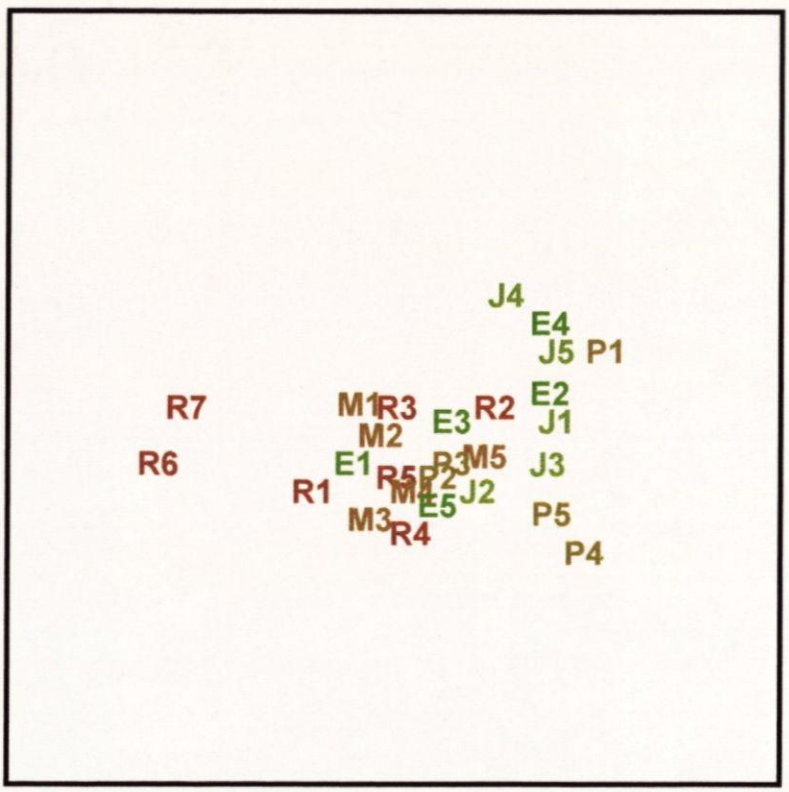


Figure 3.3 Ordination by correlation based PCA of environmental variables from Fal Estuary creeks 1993

Sediment % organic matter and % fines, pore water salinity and pH.

Community Structure:

Univariate measures

Species abundances are shown in Table 3.1, 3.2 and Appendix 3. Twenty six species, covering 18 families, 5 classes and 3 phyla were recorded. The dominant fauna were from the class Polychaeta. Univariate measures of community structure are shown in Table 3.3 and Figure 3.4. Numbers of species (*S*) are lower in Restronguet (8) and Pill (7) in 1992 and in Restronguet in 1993, but significantly higher in Percuil (16 and 15 in 1993 and 92 respectively). Total number of individuals (*A*) increase dramatically in St. Just and Percuil in 1992 and in Percuil in 1993 *i.e.* with decreased metal concentration. Species richness (*d*) in 1992 is highest in Percuil and in the most polluted creeks Restronguet and Mylor. In 1993 *d* decreases down the gradient but it is not significantly different between Restronguet, Mylor and Pill, and not between St. Just and Percuil. Evenness (*J*) and species diversity (*H'*) are also greatest at the extremes of the metal contamination gradient in 1992 and in Restronguet in 1993. Hence, a pattern that can be related to the gradient in sediment metal concentrations is not clearly revealed.

	1991		1992		1993	
RESTRONGUET						
	Paranais littoralis	554	*Nereis diversicolor*	185	*Nereis diversicolor*	39
	Nereis diversicolor	39	*Nephtys hombergi*	12	*Nephtys hombergi*	33
	Nephtys hombergi	38	*Tubificoides benedeni*	153	*Tubificoides benedeni*	23
	Pygospio elegans	15	*Pygospio elegans*	18	*Streblospio shrubsoli*	9
	Limopontia depressa	1	*Streblospio shrubsoli*	13	*Pygospio elegans*	4
	Mysid indet.	14	*Phyllodoce mucosa*	1	*Cerastoderma edule*	3
	Streblospio shrubsoli	1	*Tharyx marioni*	16	*Mysid indet.*	1
	Carcinus maenas	1	*Spio martinensis*	5	*Amphipoda indet.*	1
			Hydrobia ulvae	4		
			Cerastoderma edule	4		
			Scrobicularia plana	4		
		663		424		113
MYLOR						
	Tubificoides benedeni	2789	*Tubificoides benedeni*	2647	*Tubificoides benedeni*	855
	Streblospio shrubsoli	440	*Streblospio shrubsoli*	589	*Streblospio shrubsoli*	126
	Manayunkia aestuarina	126	*Hydrobia ulvae*	486	*Hydrobia ulvae*	70
	Nephtys hombergi	37	*Nephtys hombergi*	175	*Nephtys hombergi*	52
	Pygospio elegans	21	*Manayunkia aestuarina*	22	*Cerastoderma edule*	10
	Mysid indet.	7	*Cerastoderma edule*	16	*Tharyx marioni*	7
	Melinna palmata	3	*Scrobicularia plana*	9	*Manayunkia aestuarina*	5
	Paranais littoralis	3	*Tharyx marioni*	7	*Nereis diversicolor*	2
	Hydrobia ulvae	3	*Pygospio elegans*	6	*Heteromastus filiformis*	2
	Ampharete acutifrons	1	*Melinna palmata*	5	*Spio martinensis*	1
	Abra prismatica	1	*Mysid indet.*	4	*Scrobicularia plana*	1
			Nereis diversicolor	3	*Macoma balthica*	1
			Abra prismatica	2		
			Ostrea edulis	1		
			Carcinus maenas	1		
		3331		3973		1132
PILL						
	Tubificoides benedeni	8888	*Tubificoides benedeni*	1403	*Tubificoides benedeni*	281
	Streblospio shrubsoli	6472	*Hydrobia ulvae*	469	*Streblospio shrubsoli*	24
	Manayunkia aestuarina	2696	*Streblospio shrubsoli*	218	*Hydrobia ulvae*	13
	Melinna palmata	16	*Nephtys hombergi*	11	*Nephtys hombergi*	8
	Pygospio elegans	124	*Capitella capitata*	246	*Cerastoderma edule*	4
	Nephtys hombergi	14	*Manayunkia aestuarina*	45	*Melinna palmata*	3
	Nereis diversicolor	4	*Scrobicularia plana*	6	*Pygospio elegans*	2
	Hydrobia ulvae	4	*Tharyx marioni*	5		
	Scrobicularia plana	4	*Melinna palmata*	3		
	Mysid indet.	3	*Nereis diversicolor*	2		
			Macoma balthica	2		
			Glycera sp.	1		
			Abra prismatica	1		
			Anenome indet.	1		
		18270		2314		335

Table 3.1 Species abundance lists 1991-1993 for Fal Estuary creeks: Restronguet, Mylor and Pill

per sediment core

(1991 data from Perryman 1992).

	1991		1992		1993	
ST. JUST						
	Tubificoides benedeni	2545	*Tubificoides benedenni*	211	*Tubificoides benedenni*	2779
	Manayunkia aestuarina	500	*Streblospio shrubsoli*	3368	*Streblospio shrubsoli*	130
	Streblospio shrubsoli	315	*Capitella capitata*	215	*Capitella capitata*	79
	Paranais littoralis	61	*Manayunkia aestuarina*	12	*Nereis diversicolor*	46
	Nephtys hombergi	40	*Nereis diversicolor*	168	*Cerastoderma edule*	31
	Cerastoderma edule	9	*Hydrobia ulvae*	35	*Hydrobia ulvae*	17
	Nereis diversicolor	7	*Cerastoderma edule*	16	*Manayunkia aestuarina*	10
	Mysid indet.	4	*Nephtys hombergi*	15	*Amphipoda indet.*	9
	Pygospio elegans	1	*Tharyx marioni*	1	*Nephtys hombergi*	8
	Macoma balthica	1	*Scrobicularia plana*	1	*Scrobicularia plana*	5
			Pygospio elegans	16	*Carcinus maenas*	1
			Paranais littoralis	6		
			Macoma balthica	2		
		3483		2987		3115
PERCUIL						
	Tubificoides benedeni	2278	*Tubificoides benedeni*	6270	*Tubificoides benedeni*	181
	Streblospio shrubsoli	307	*Streblospio shrubsoli*	3071	*Streblospio shrubsoli*	814
	Manayunkia aestuarina	56	*Manayunkia aestuarina*	2810	*Tharyx marioni*	2880
	Nephtys hombergi	33	*Hydrobia ulvae*	295	*Manayunkia aestuarina*	104
	Cerastoderma edule	12	*Pygospio elegans*	73	*Pygospio elegans*	93
	Pygospio elegans	10	*Tharyx marioni*	59	*Hydrobia ulvae*	51
	Hydrobia ulvae	6	*Melinna palmata*	57	*Capitalla capitata*	44
	Paranais littoralis	3	*Nephtys hombergi*	34	*Cerastoderma edule*	36
	Mysid indet.	3	*Scrobicülaria plana*	32	*Ampharete acutifrons*	18
	Tharyx marioni	2	*Macoma balthica*	32	*Nephtys hombergi*	16
	Scrobicularia plana	2	*Spio martinensis*	20	*Spio martinensis*	13
	Anenome indet.	1	*Cerastoderma edule*	17	*Nereis diversicolor*	6
			Phyllodoce mucosa	8	*Melinna palmata*	4
			Mysid indet.	3	*Anenome indet*	2
			Anenome indet.	1	*Scrobicularia plana*	1
					Mysid indet.	1
		2713		12782		4229

Table 3.2 Species abundance lists 1991-1993 for Fal Estuary creeks

St. Just and Percuil

(1991 data from Perryman 1992).

	S	*A*	*d*	*H'*	*J*
Restrong.	8 ± 2.0	113 ± 64	1.48 ±	1.55 ±	0.74 ± 0.1
Mylor	12 ± 1.5	1130 ± 140	1.56 ±	0.908 ±	0.36 ± 0.04
Pill	7 ± 2.2	335 ± 290	1.03 ±	0.677 ±	0.34 ± 0.02
St. Just	11 ± 2.0	3120 ± 580	1.24 ±	0.529 ±	0.22 ± 0.18
Percuil	16 ± 1.5	4230 ± 1100	1.8 ±	1.45 ±	0.52 ± 0.11

Table 3.3a. Basic descriptive variables for macrofauna samples from creeks in Fal Estuary: number of species *S*, number of individuals *A*, species diversity *H'*, evenness *J* and richness *d* (±S.D) 1992.
(n = 5, with the exception of Restronguet where n = 7).

	S	*A*	*d*	*H'*	*J*
Restrong.	11 ± 1.5	424 ± 130	1.65 ± 0.5	1.56 ± 0.20	0.65 ± 0.21
Mylor	15 ± 2.1	3970 ± 620	1.69 ± 0.6	1.06 ± 0.24	0.39 ± 0.17
Pill	14 ± 2.2	2310 ± 154	1.68 ± 0.2	1.21 ± 0.18	0.45 ± 0.20
St. Just	13 ± 0.55	2980 ± 380	1.5 ± 0.4	1.08 ± 0.14	0.42 ± 0.19
Percuil	15 ± 0.50	12800 ±1900	1.48 ± 0.8	1.26 ± 0.18	0.46 ± 0.22

Table 3.3b Basic descriptive variables for macrofauna samples from creeks in Fal Estuary: number of species *S*, number of individuals *A*, species diversity *H'*, evenness *J* and richness *d*. 1993
(+ S.D)(n = 5, with the exception of Restronguet where n = 7).

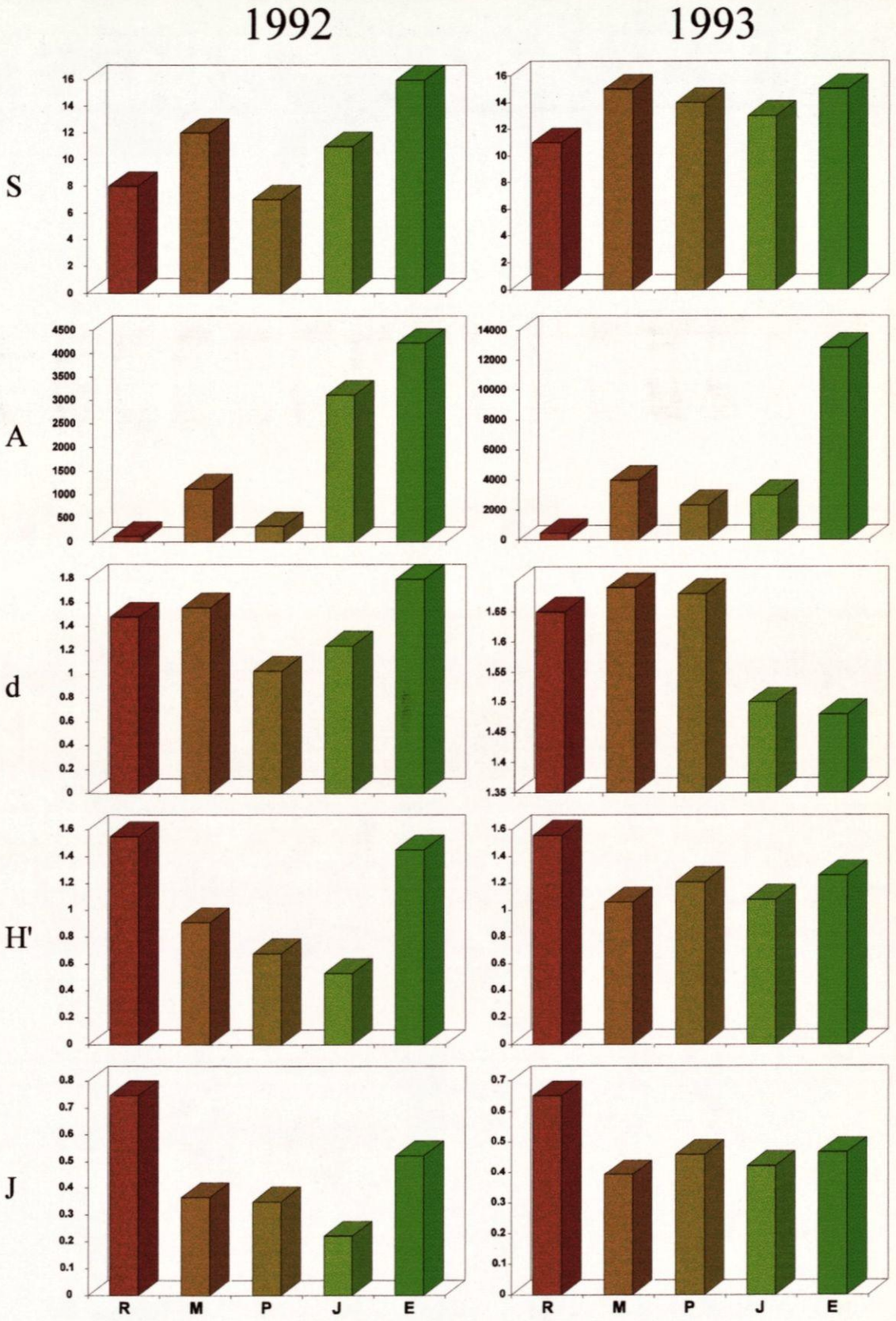


Figure 3.4 Univariate measures for Fal Estuary macrofauna

1992 (left column) and 1993 (right column)
(a) Number of species, S (b) Number of individuals, A (c) Richness, d
(d) Species diversity, H' and (e) Evenness, J.

Multivariate analyses

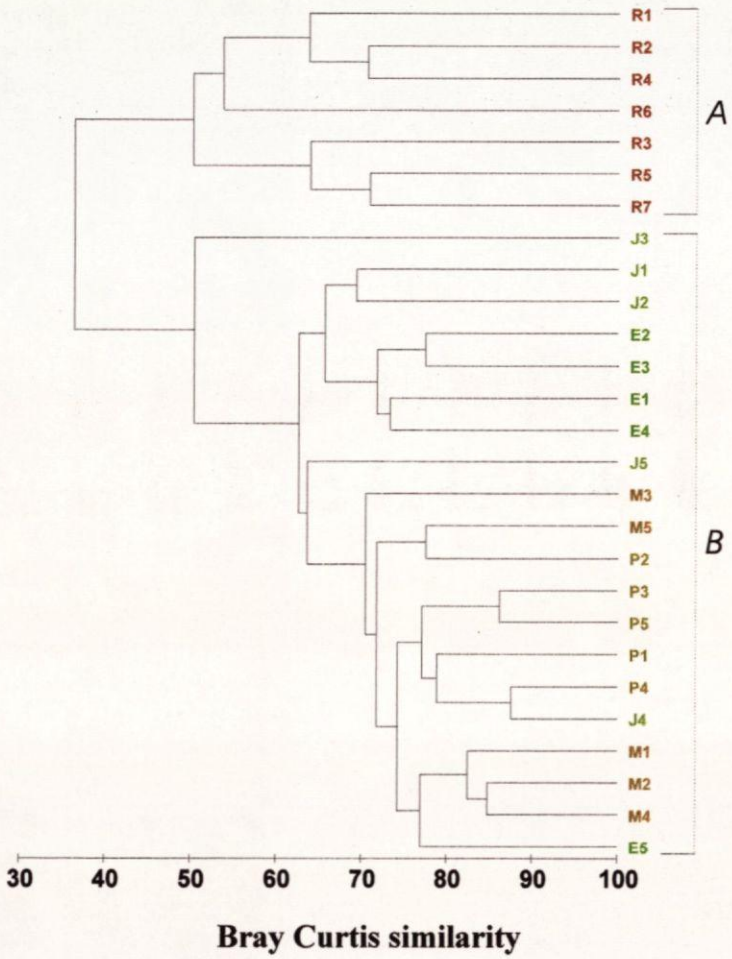
Single year data

The dendrograms (Figures 3.5a and 3.6a) show Restronguet (cluster A) to be separated clearly from the other four creeks (cluster B) at a 45% similarity level in 1992 and 1993.

The MDS configurations (Figure 3.5b and 3.6b) for 1992 and 1993 show that the five creeks each have distinct macrofaunal assemblages. ANOSIM confirms that the communities in all creeks differ significantly ($p < 5\%$) from each other. They show a similar pattern to that of the PCA plots of metal data (see Figure 3.2). The positions on the MDS plots for St Just, Pill, Mylor and Percuil are more clustered and slightly set apart from Restronguet Creek. In 1992 the creeks are separated by their community structure with the exception of Pill which is indistinguishable from Mylor and St. Just. Samples within some specific creeks, *e.g.* St Just and Restronguet, are also ordered, with samples from the head of the creek furthest away from all other sites. Stress values are very low (0.11 and 0.10 for 1993) showing the goodness of fit.

The values for the index of dispersion (Table 3.4) show Restronguet to be the most dispersed in both 1992 and 1993 indicating variability which may be a sympton of stress (Warwick & Clarke 1993). However, the least contaminated site, Percuil, and the penultimate one, St. Just, also show high values of dispersion. Thus, the variability in terms of dispersion is not apparently consistent with the metal gradient.

(a)



(b)

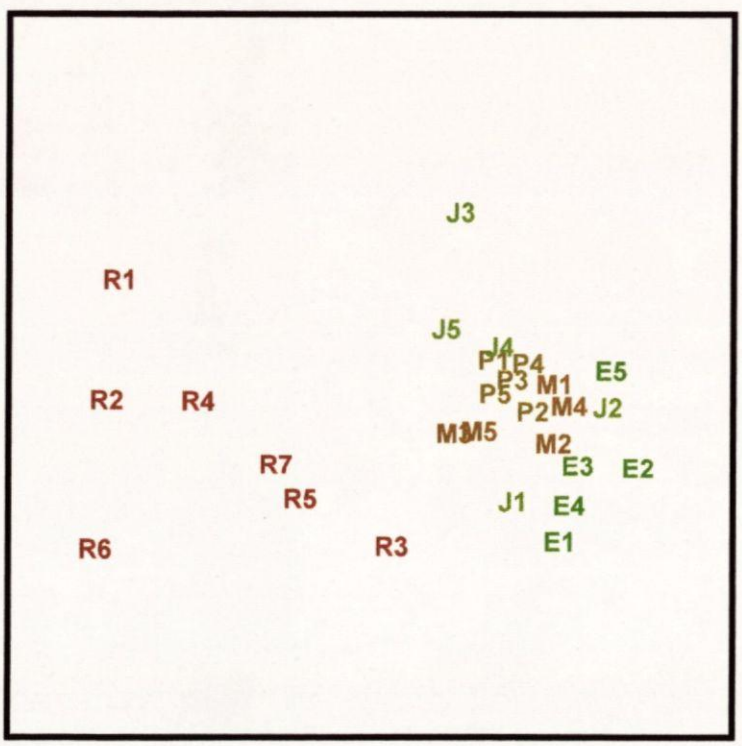
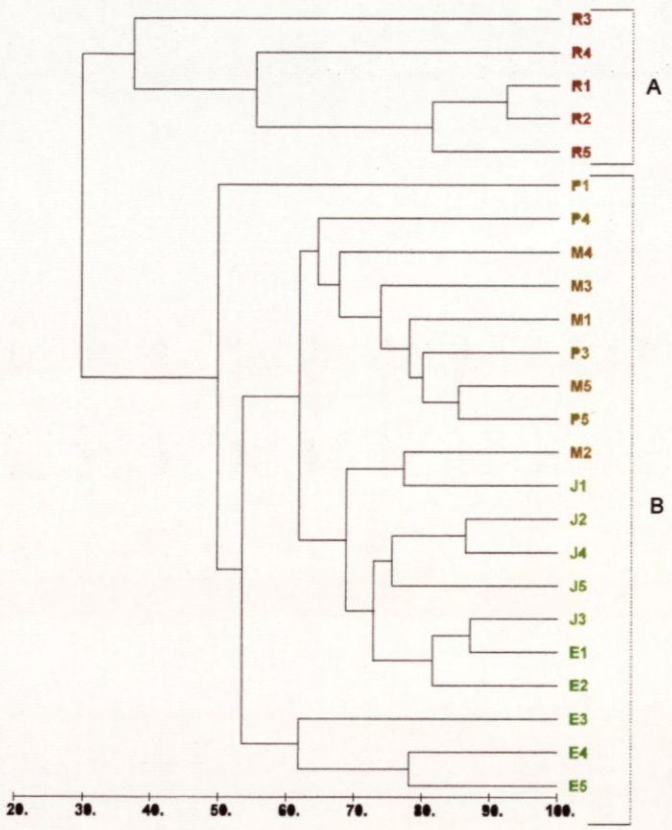


Figure 3.5. Macrofauna abundance data 1992 (4th root transformed): (a) Dendrogram (b) MDS plot (stress 0.12).

(a)



(b)

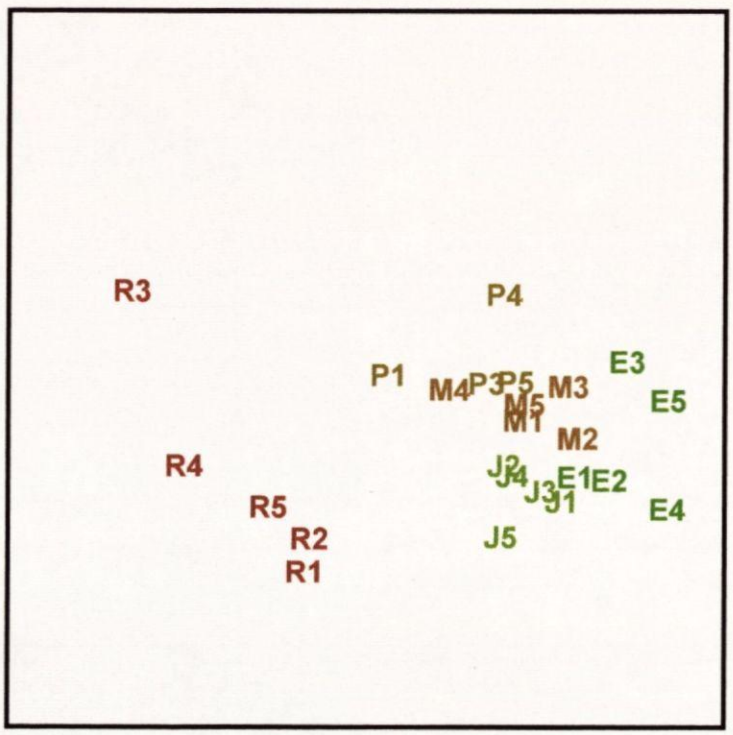


Figure 3.6 Macrofauna abundance data 1993 (4th root transformed): (a) Dendrogram (b) MDS plot (stress 0.11).

	1993	1992
Restronguet	1.34	1.45
Mylor	0.66	0.45
Pill	1.24	0.45
St Just	0.45	1.36
Percuil	0.94	1.45

Table 3.4 Relative index of multivariate dispersion for Fal Estuary creeks 1992 and 1993.

Relating community to environment

The variation in community structure can be explained by the measured environmental variables revealed by BIOENV analyses, summarised in Table 3.5. The highest rank correlation with macrofauna abundance data occurs with the single variable zinc (0.443 and 0.502) closely followed by copper (0.402 and 0.469) confirming that the differences in macrofauna communities are best explained by levels of heavy metals in the sediments. Adding metals together increases the correlation between metals and biota *e.g.* copper and zinc (0.505) and cadmium and zinc (0.450) for 1993 data. In comparison, natural environmental variables are less well correlated *e.g.* percentage organic matter has a value of 0.304 against 1993 community data.

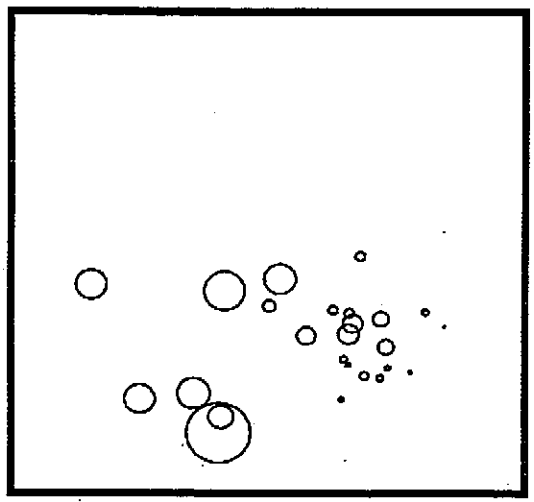
1993			
Number of variables			
1	**Zinc**	Copper	% organic matter
	0.443	0.402	0.304
2	Cadmium and Zinc	Cadmium and Cobalt	Cadmium and Copper
	0.450	0.435	0.431
3	Cadmium,Cobalt and	Cadmium,Copper and	
	Zinc 0.452	Zinc 0.444	
1992			
Number of variables			
1	**Zinc**	Copper	% organic matter
	0.502	0.469	0.329
2	Copper and Zinc 0.505	Copper and Iron	
		0.466	
3	Copper, Iron and Zinc		
	0.484		

Table 3.5 Summary of results from BIOENV. Combinations of variables, n at a time, giving the highest rank correlations between biotic and abiotic similarity matrices

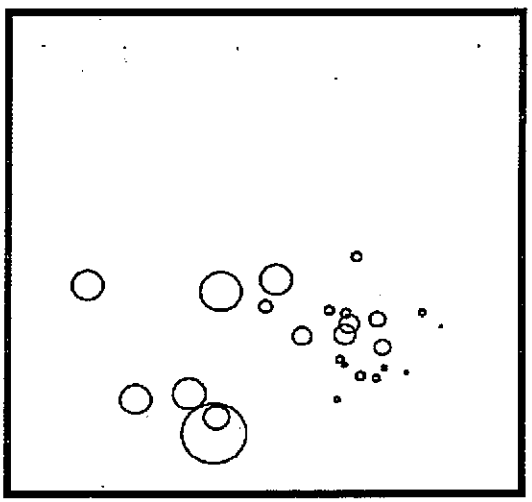
(lower correlations omitted).

Such data are visually represented by overlaying MDS plots with the measured metal concentrations to illustrate the relationship between metal levels and community structure and to obtain indications of the variables that correlate with the group differences. Figures 3.7-3.10 show the MDS configurations for 1992 and 1993 with circles relating to concentrations of of natural and contaminant variables superimposed. The macrofauna in each creek can be distinguished on the basis of zinc, copper, iron, cobalt, cadmium and % organic matter levels in the sediments, with the community in Restronguet Creek clearly separated from the rest, and the samples from Mylor, Restronguet and Percuil roughly ordered horizontally along the ordination according to metal concentrations. Percentage fines, salinity, pH and manganese show no relationship with community composition.

(a) Zinc



(b) Cadmium



(c) Cobalt

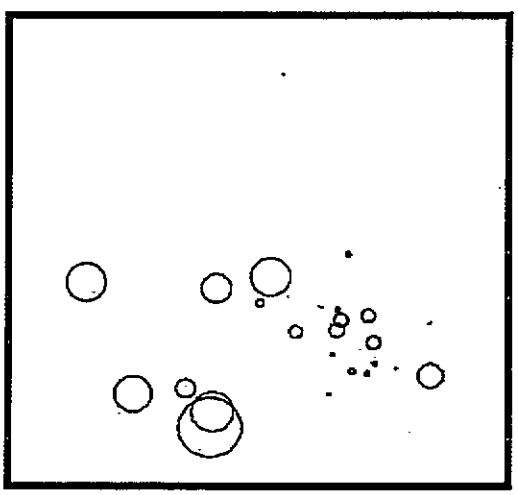
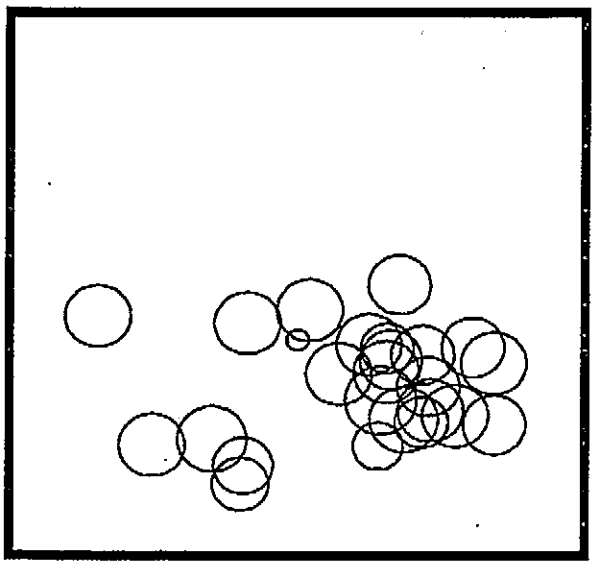
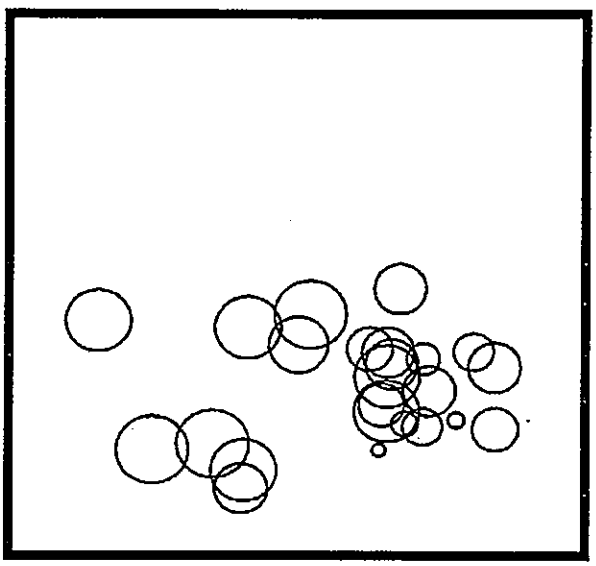


Figure 3.7. MDS ordination plots 1993
macrofauna data: Sediment metal content superimposed

(a) Salinity



(b) pH



(c) % Fines

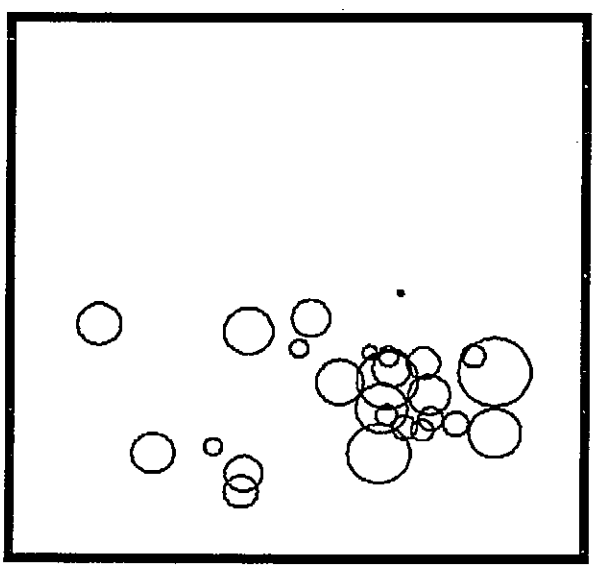


Figure 3.8 MDS ordination of macrofauna abundance data 1993 (4th root transformed): Environmental variables superimposed.

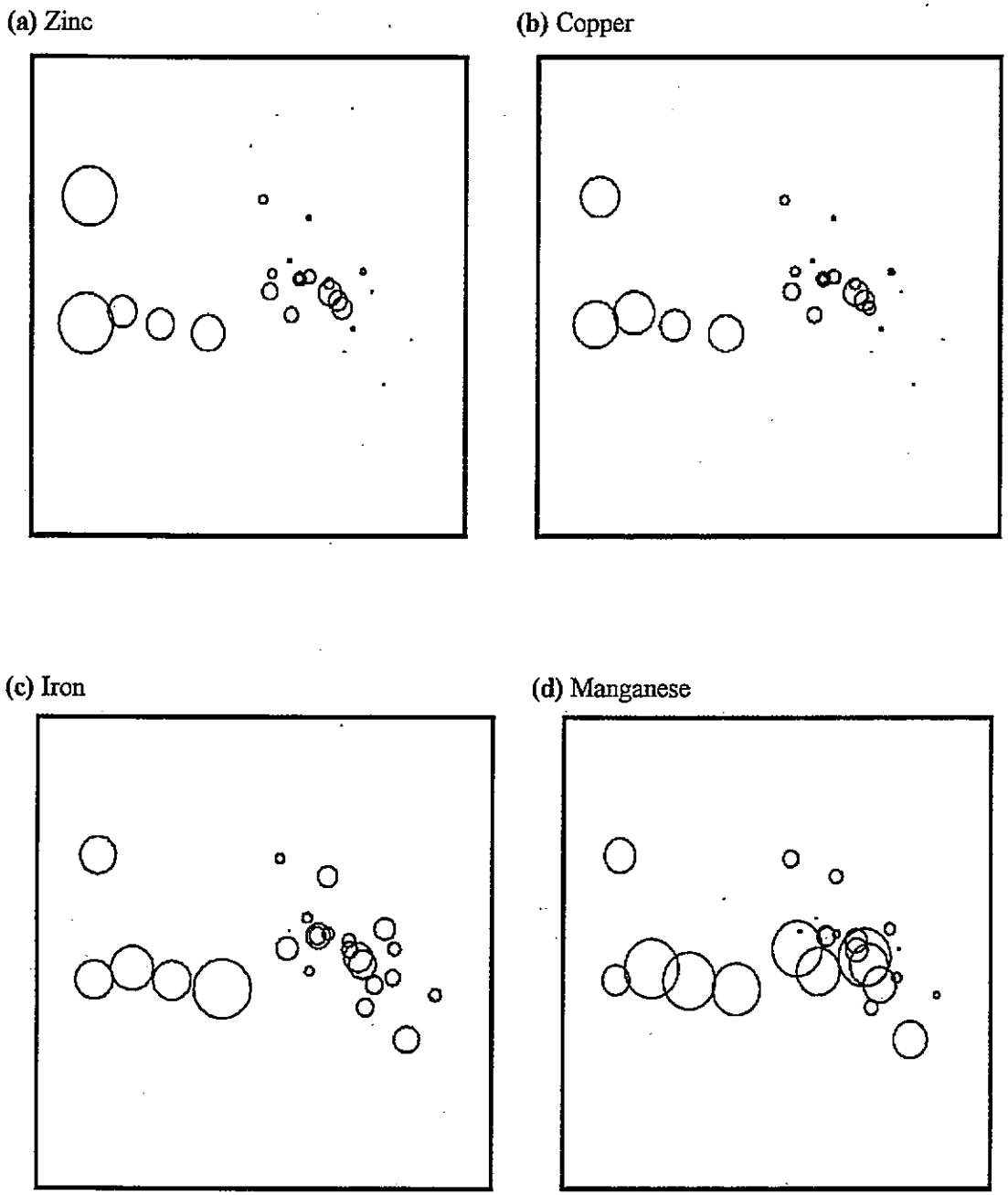
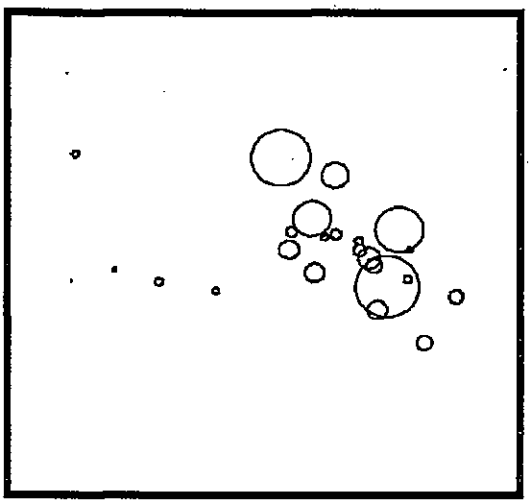


Figure 3.9 MDS ordination of Fal Estuary macrofauna abundance data (4th root transformed) 1992: Sediment metals superimposed.

(a) Percentage organic



(b) Percentage fines

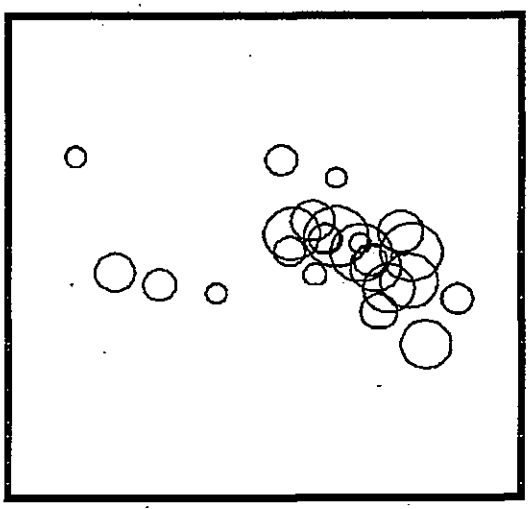


Figure 3.10. MDS ordination of macrofauna abundance data 1992 (4th root transformed): Environmental variables superimposed.

Discrimination between sites on basis of the organisms present

SIMPER analyses (Table 3.6 and 3.7) of transformed abundance data identifies the species which contribute to the dissimilarity between creeks and years. Additional information is provided by the bar charts in Figure 3.11-3.13, Table 3.1 and 3.2, and additional facts on species characteristics in Appendix 2.

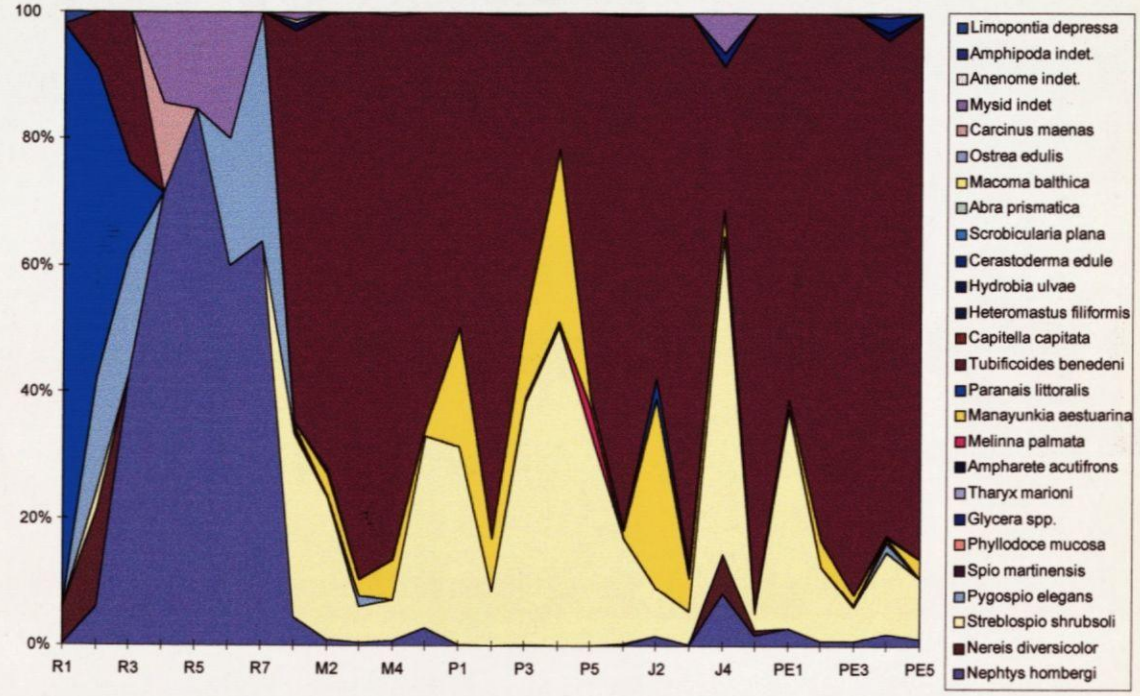
Species	R		M		P		J		E
Nephtys hombergi	17.3	<	35.0	>	22.4	>	3.0	<	6.8
Tubificoides benedeni	7.6	<	529.4	>	280.6	<	422.6	>	1254.0
Streblospio shrubsolii	1.86	<	117.8	>	43.6	<	71.6	<	614.2
Cerastoderma edule	0.6	<	3.2	<	9.2	>	3.2	<	3.4
Scrobicularia plana	0.6	<	1.8	>	1.2	<	2.2	<	6.4
Tharyx marioni	0.9	<	1.4	>	1.0				11.8
Pygospio elegans	2.6	>	1.2				1.2	<	14.6
Nereis diversicolor	26.4	>				>	13.6		
Phyllodoce mucosa	1.6								1.6
Hydrobia ulvae			97.2	>	93.8	>	7.0	<	59.0
Manayunkia aestuarina			4.4	<	9.0	<	24.2	<	562.0
Abra prismatica			0.4						
Mysid *indet*			0.8						
Melinna palmata					0.6	<		<	11.4
Capitella capitata							43.0		
Spio martinensis									4.0

Table 3.6 Summary of similarity terms (SIMPER) analysis. Differences (< and >) in average abundances of species contributing to differences between consecutive creeks along the metal gradient 1992. A cut-off of 100% similarity was applied.

Species	R		M		P		J		E
Nephtys hombergi	4.7	<	10.4	>	1.6	-	1.6	>	3.2
Tubificoides benedeni	3.3	<	171.0	>	56.2	<	555.8	>	363.6
Streblospio shrubsolii	1.3	<	25.2	>	4.8	<	26.4	<	228.4
Nereis diversicolor	5.6	<				<	9.2	>	1.2
Cerastoderma edule			2.0	>	0.8	<	6.2	<	7.2
Tharyx marioni			1.4	<				<	176.0
Hydrobia ulvae			14.0	>	2.6	<	3.4	<	10.2
Manayunkia aestuarina			1.0	<				<	20.8
Capitella capitata							15.8	>	8.8
Ampharete acutifrons									3.6
Melinna palmata									0.8
Spio martinensis									2.6

Table 3.7 Summary of similarity terms (SIMPER) analysis. Differences (< and >) in average abundances of species contributing to differences between consecutive creeks along the metal gradient 1993. A cut-off of 100% similarity was applied.

(a)



(b)

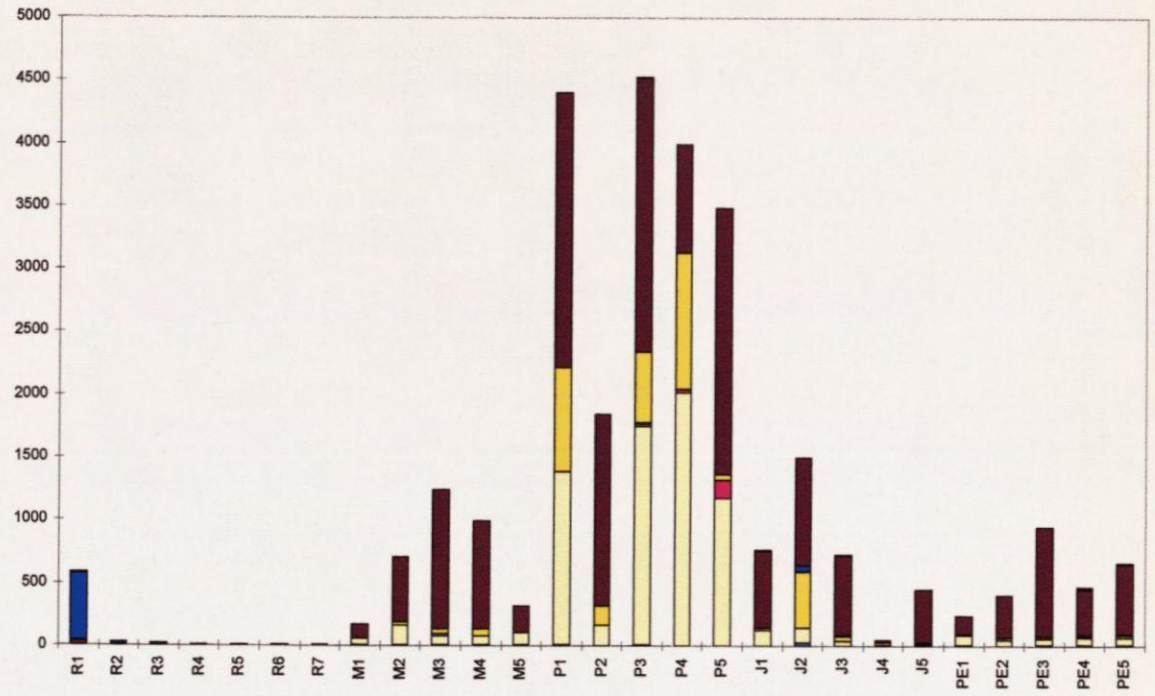
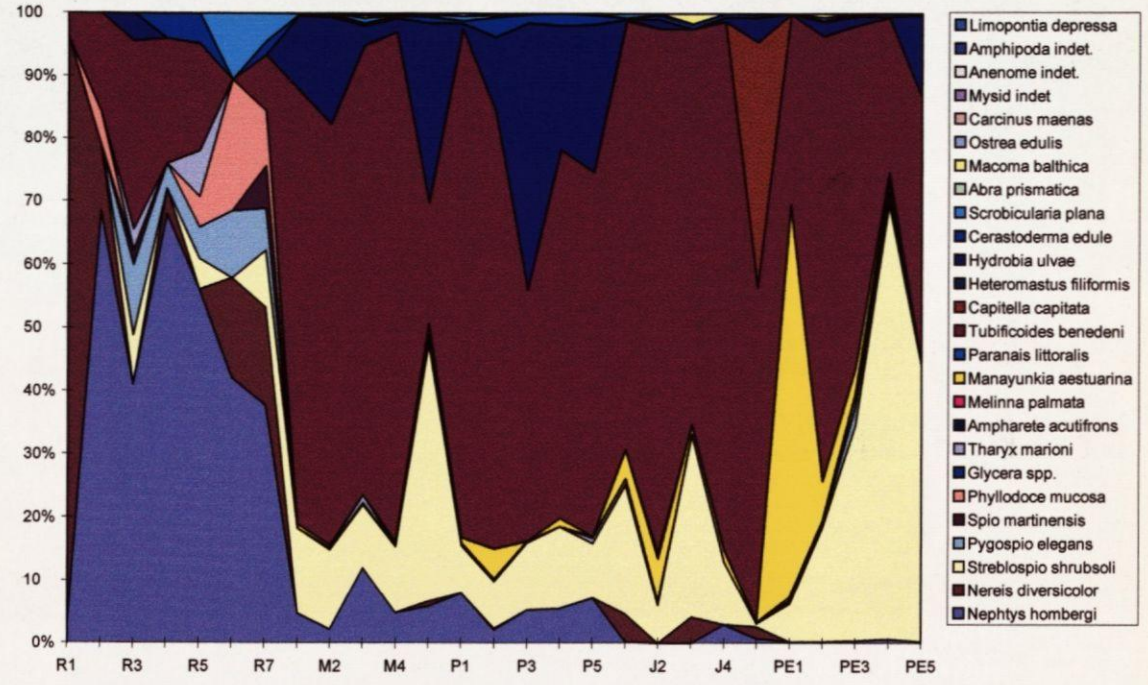


Figure 3.11 Species abundance data 1991

(a) Percentage abundance per site (b) Total numbers per site

(a)



(b)

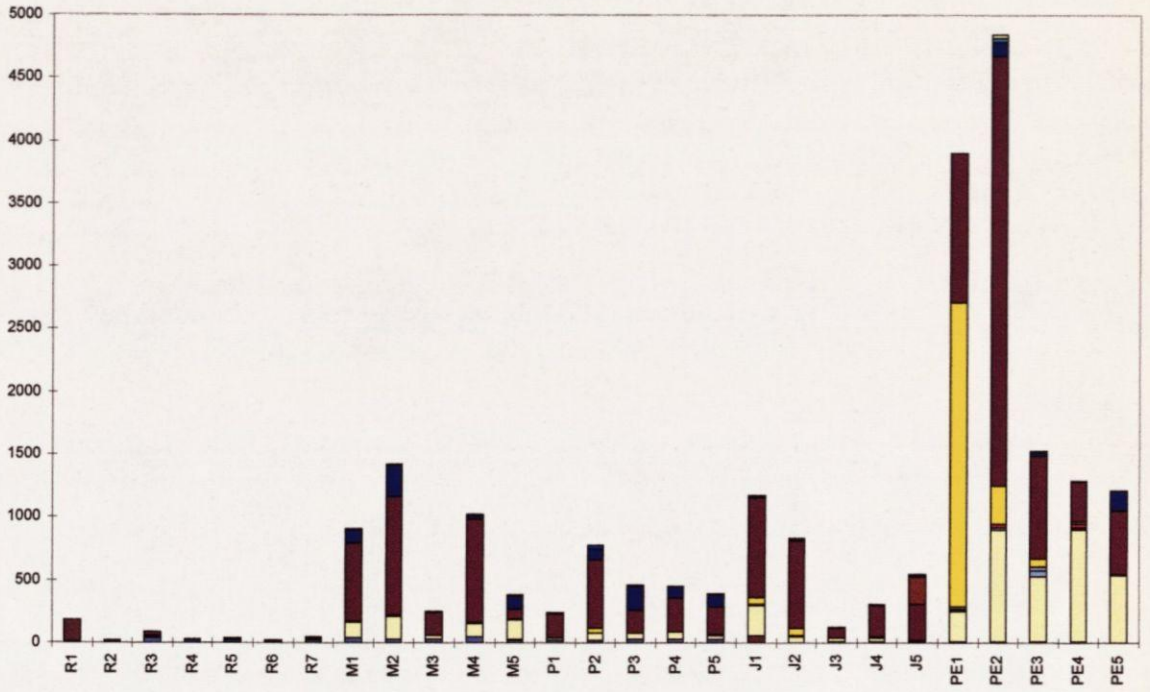
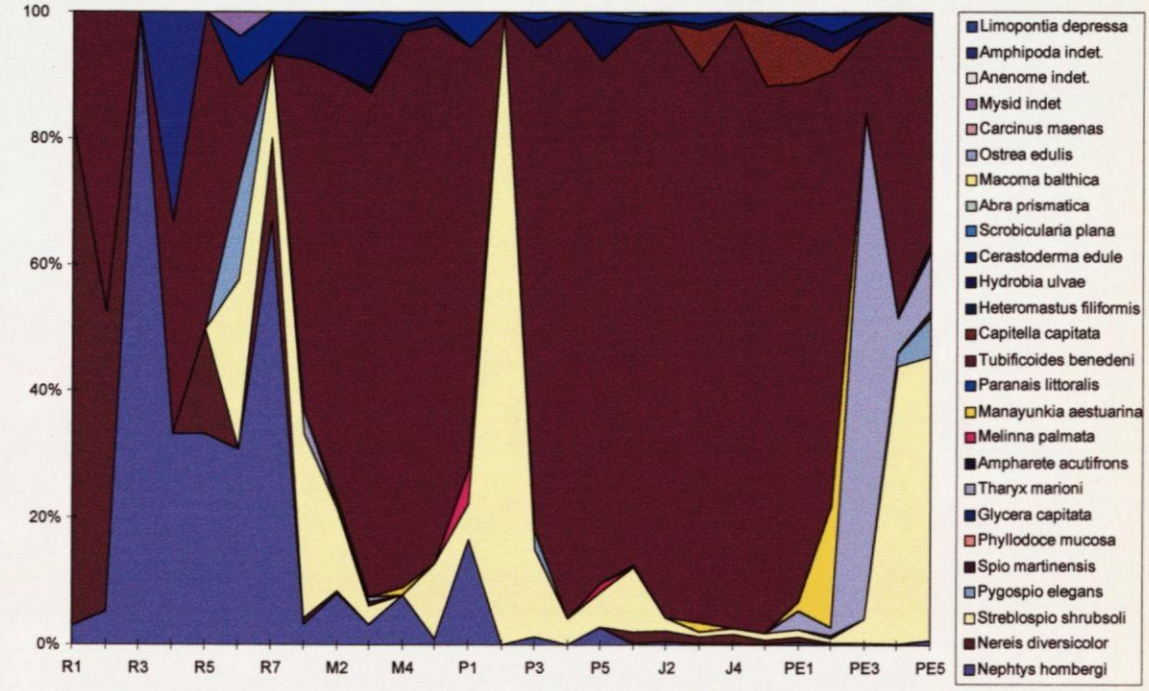


Figure 3.12 Species abundance data 1992

(a) Percentage abundance per site (b) Total numbers per site

(a)



(b)

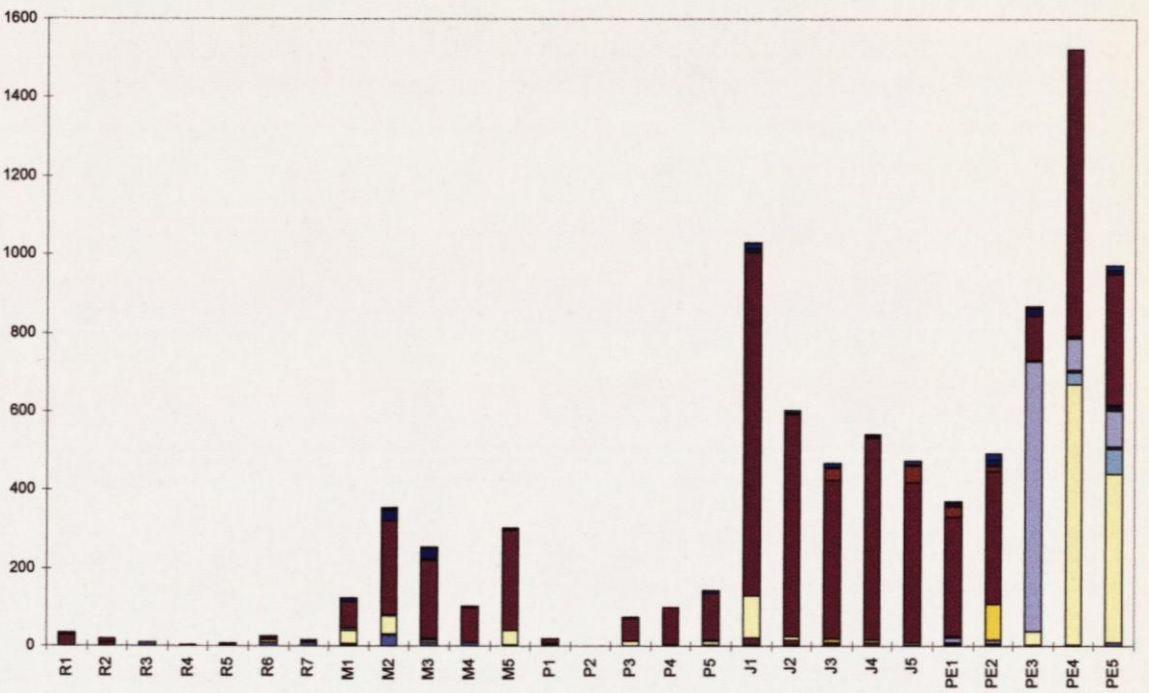


Figure 3.13 Species abundance data 1993

(a) Percentage abundance per site (b) Total numbers per site

With the exception of Restronguet the creeks are dominated numerically by the oligochaete *Tubificoides benedeni*. The community in Restronguet Creek is very different from the others being dominated by the polychaetes *Nephtys hombergi* and *Nereis diversicolor*. Restronguet is also distinguished by the absence of *Tharyx marioni, Manayunkia aestuarina, Melinna palmata, Capitella capitata, Ampharete acutifrons* and the bivalves *Scrobicularia plana and Macoma balthica*. There is a general increase in abundance of a broad range of species in creeks with lower sediment metal concentrations, and a reduction in numbers and importance of *N. diversicolor* and *N. hombergi*. St Just and Percuil are separated from the others by the presence of relatively large numbers of *Tharyx marioni* and *Capitella capitata*, suggesting organic enrichment (Pearson and Rosenberg 1978). This prompted an investigation into the bacteriological status, the results for which are presented in Table 2.5 (Chapter 2). They show levels of faecal streptococci and *E. coli* to be extremely low.

Other common species ubiqutously present throughout the creeks greatly differ in their abundance, such as *Manayunkia aestuarina* which is most abundant in Percuil creek and *Hydrobia ulvae* which is most abundant in Mylor. Many species were absent from Pill Creek in 1993 *e.g. N. diversicolor, M. aestuarina,* or present in very much lower numbers e.g. *H. ulvae.*

Taxonomic levels and discrimination of creeks

MDS ordinations of the macrofauna abundance data from 1992 (Figure 3.14) indicate that as abundances are aggregated to higher taxonomic levels the overall patterns of community structure are visually retained. The plots from the highest taxonomic levels, phylum and class, have the lowest stress levels (0.1 and 0.08) which reflect the extent to which the MDS preserves inter-sample relationships in a 2-dimensional plot due to a reduction in the number of taxa.

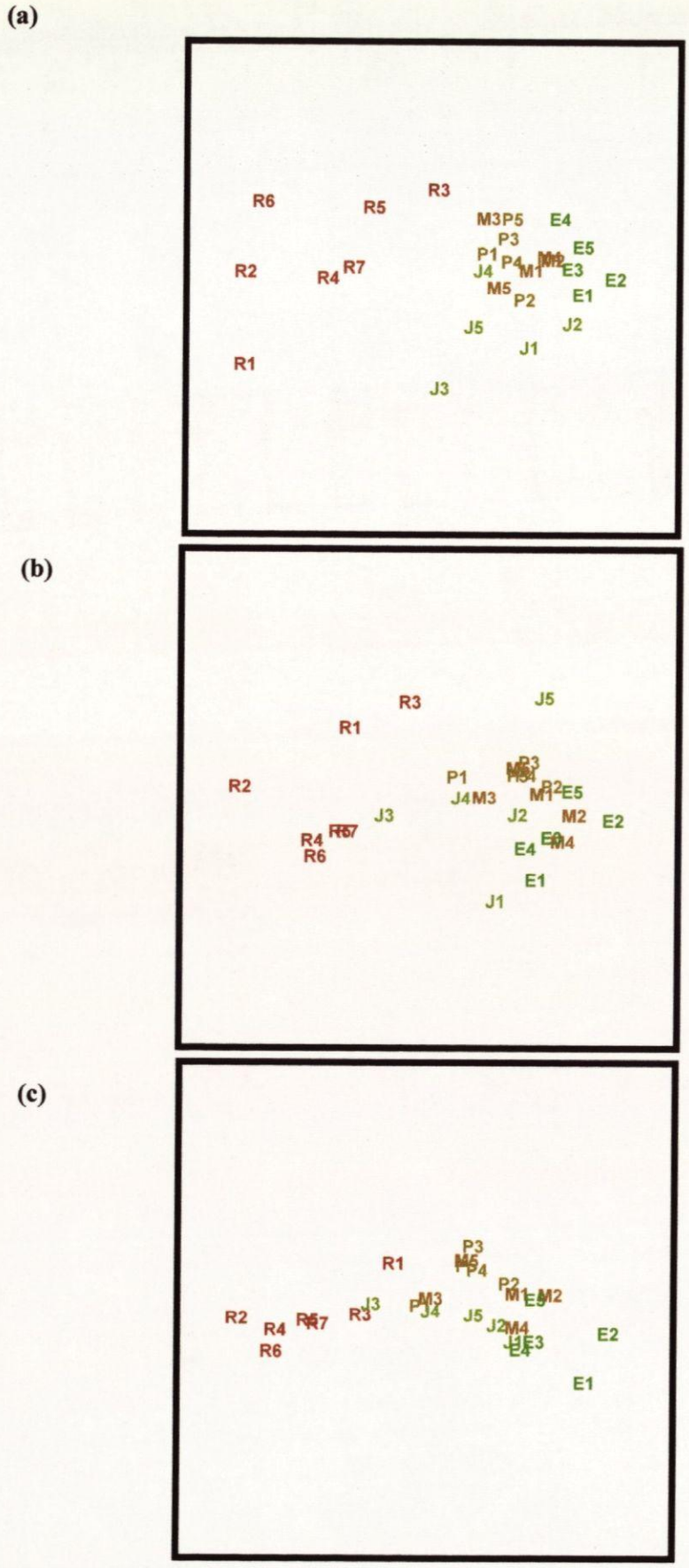


Figure 3.14 MDS ordination plots for Fal Estuary macrofauna (1992) aggregated to various taxonomic levels (a) Family (b) Class (c) Phylum.

RELATE, which tests the significance of agreement between multivariate patterns, was used to substantiate these data (Table 3.8). Results of tests for differences between the similarity matrices underlying these ordinations confirm that the matrices are highly correlated at all taxonomic levels, indicated by the high values ranging from 0.750 to 0.953. This shows that aggregating to family, class and even phylum loses little or no information. For matrices, correlations decrease only slightly as taxonomic levels increase; species are more highly correlated with family (0.953) than they are with class, and more with class than they are with phylum (0.845). The relationships between matrices derived from abundances of macrofauna phyla and lower taxonomic levels of family are marginally less clear (0.750) (as Somerfield & Clarke 1995 found with their analysis of Fal data).

	Family	**Class**	**Phylum**
Species	0.953	0.865	0.845
Family		0.852	0.750
Class			0.855

Table 3.8 Pair-wise Spearman rank corelations between similarity matrices, derived from macrofauna species abundance data (1992), aggregated to a range of taxonomic levels.

All correlations are significantly different from zero, by a permutation test (RELATE), at least $p < 1\%$

Time series data 1991-1993.

For this section, all data are averaged for the sites in each creek to give an overall average creek value, and prepublished data is used for 1991 (from Perryman 1992). The MDS plot for macrofauna data (Figure 3.15) represent data for the three years combined and show a shift of the communities over this period vertically up the plot (with the exception of Percuil) as indicated by arrows. Over these three years, the patterns are not consistent with the PCA plot for sediment metal levels for creek averaged data (Figure 3.16) which remain constant for the duration.

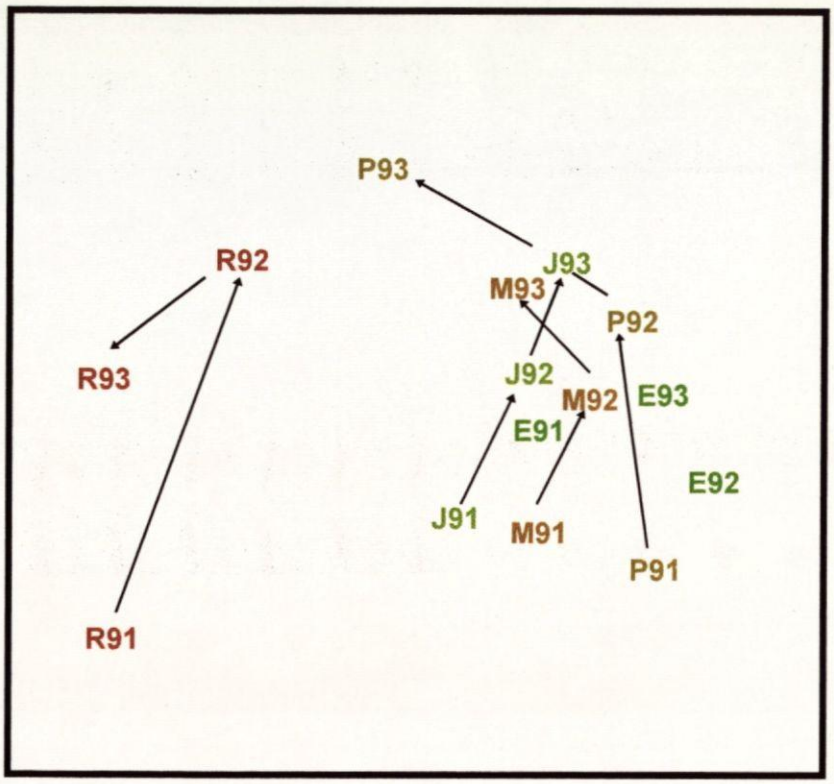


Figure 3.15 Macrofauna time series 1991-1993: MDS ordination of 4th root transformed abundance data (stress 0.12). Arrows denote temporal shift in creek MDS positions.

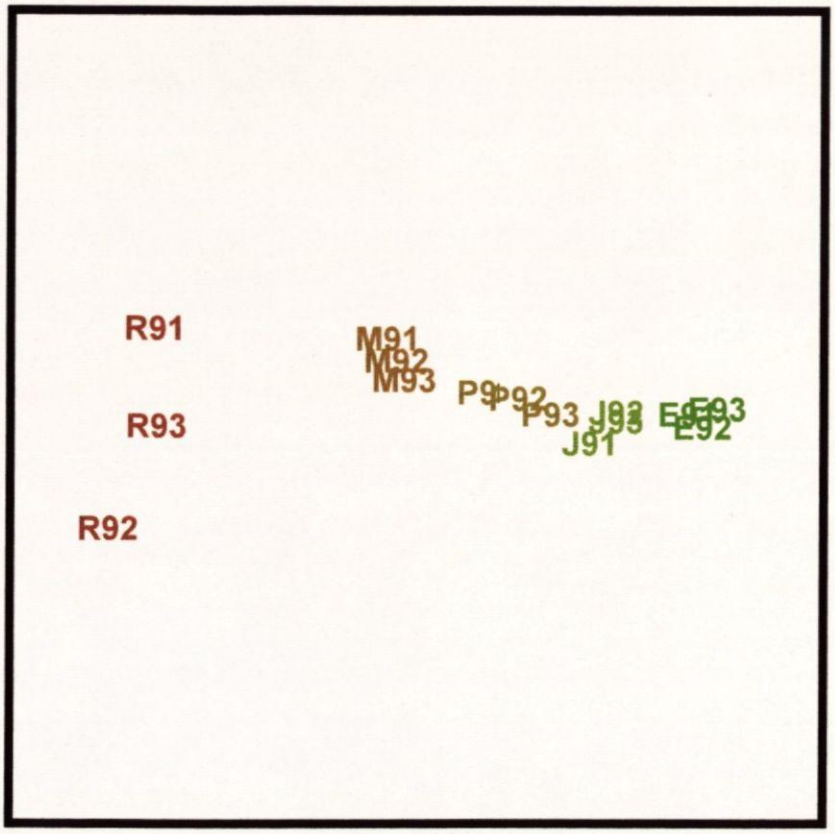


Figure 3.16 Heavy metals time series data 1991-1993: PCA ordination of sediment metal content (stress 0.01).

SIMPER results (Table 3.6) showed that these changes were the result of changes in the relative abundances of certain species. (e.g. there was a decrease in *Tubificoides benedeni, Manayunkia aestuarina, Streblospio shrubsolii,* and *Paranais littoralis* and an increase in *Tharyx marioni, Nereis diversicolor, Cerastoderma edule, Scrobicularia plana* and *Melinna palmata*) rather than the gain or loss of species, with the exception of the gain of minimal numbers of *Phyllodoce mucosa, Glycera* spp. and *Heteromastus filiformis*, and this is also seen in the series of bar charts (Figure 3.10 and 3.11). There is an increase in bivalve molluscs in 1993 and 1992 over 1991, with the exception of Restronguet. The two-way ANOSIM test for differences between sites was significant ($p < 1\%$) and for between years was also significant ($p < 1\%$).

Species	1991		1992		1993
Tubificoides benedeni	660.3	>	498.8	>	229.9
Streblospio shrubsoli	301.4	>	169.8	>	57.2
Manayunkia aestuarina	135.2	>	119.9	>	4.8
Nephtys hombergi	6.05	<	16.9	>	4.3
Nereis diversicolor	1.6	<	8.2	>	3.3
Pygospio elegans	2.7	<	3.9	-	3.9
Cerastoderma edule	0.8	<	3.9	>	3.3
Hydrobia ulvae	0.5	<	15.5	>	6.1
Mysid indet	0.8	>	0.3	>	0.1
Melinna palmata	6.6	>	2.6	>	0.3
Scrobicularia plana			2.4	>	0.3
Tharyx marioni			3.5	<	35.5
Paranais liitoralis	18.5				
Abra prismatica			0.1		
Macoma balthica			1.4		
Phyllodoce mucosa			0.6		
Spio martinensis			0.9		
Capitella capitata					4.9

Table 3.9 Summary of similarity terms (SIMPER) analysis. Differences (< and >) in average abundances of species contributing to dissimilarities between consecutive years. A cut-off of 100% similarity was applied.

DISCUSSION

Observations of and comments on the community structure of the creeks.

Multivariate analyses show that there are clear differences in community composition associated with the heavy metal pollution gradient occurring up to 5 km away from the source. The five creeks each have significantly different macrofaunal assemblages. Restronguet is very different to the other four creeks which are clustered more closely together. However species diversity, richness and evenness are not consistent with the metal gradient and show the lower sensitivity of univariate measures. Various factors can be examined when considering these faunistic observations.

Adaptation and tolerance.

There is a surprising abundance of organisms present throughout the creeks considering the fact that it is such a contaminated estuary. However, many of the Fal Estuary organisms are historically adapted to high concentrations of metals in the sediment environment (Bryan & Gibbs 1983, Hately *et al.* 1989). They exhibit tolerance mechanisms to heavy metals as shown by previous studies on *Nereis diversicolor* (Grant *et al.* 1989) which stores zinc in its jaws for strengthening purposes; the ampharetid *Melinna palmata* which accumulates copper in its gills which has the defensive function of reducing palatability of the worm to predators; and the cirratulid *Tharyx marioni* which has a high level of arsenic in its feeding palps. These latter two species were not common in Restronguet Creek. *Carcinus maenas* has decreased permeability to zinc which allows more time for the metal to be excreted and thus regulated and so these mechanisms ameliorate the predicted effects of metal

contamination (Bryan & Langston 1992). Blackening of adult *N. diversicolor* and *Nephtys hombergi* was observed, especially in Restronguet Creek, resulting from uptake of metals following close contact with contaminated sediment especially at low salinity.

Despite these tolerances, a pattern remains in the community structure which has been shown to be persistently pertaining to the metal gradient, perhaps because some species have remained unable to tolerate very high metal levels; Bivalve molluscs are poor regulators of heavy metals and they were virtually absent from the most contaminated creek, Restronguet. Some species are common and often dominant in other creeks *e.g. Tubificoides benedeni* and *Streblospio shrubsolii* were absent from Restronguet Creek suggesting that they may not be able to adapt to such extremes of pollution. The difference in toxic effects between species may be because most species are highly selective in their choice of diet and only some of the available food is toxic and therefore not all of them experience toxic effects. However, insufficient information is available on diet and digestive physiology of most soft bottom species (Olsgard 1993).

Life history strategy.

Epibenthic species may be affected differently from endobenthic species. Epibenthic species exist in all the creeks, but there were no endobenthic species found in Restronguet Creek. Vertical distribution of organisms in the sediments and their recruitment rates vary. In mud most species are found in the top 1 cm of the sediment (Palmer 1984). This facilitates rapid population recovery in surface sediments where contaminant levels may decline rapidly soon after the cessation of pollution. Some species however penetrate deeper into the sediment where contaminants persist longer, and therefore these assemblages may take much longer to recover. Sediment samples on which the environmental data were based

were obtained from surface scrapes only, therefore the relevance of the environmental parameters to macrofauna living deeper than 2 cm should be treated cautiously.

Sediment characteristics.

Sediment particle size is a most important ecological factor influencing the distribution of polychaetes. The sediment characteristics of estuaries can be affected by natural and man made events; heavy river run-offs can deposit new sediments or re-suspend existing ones. Discharge of industrial effluents can alter physical as well as chemical characteristics of the sediments. In this specific case, the millions of gallons of spill waters from the tin mine in early 1992 could either have had a scouring effect on the benthos, or they could have deposited particulate matter. However, Olsgard (pers. comm.) did not find any faunal differences after the flooding of the River Gomma, Norway. He found the benthos could tolerate several millimetres per year without traceable effects on the fauna. Sedimentation rates in the Restronguet Creek are not in excess of this rate (NRA pers. comm.). But, looking to the future, the construction of the water treatment plant on the Carnon river could alter the existing current flow pattern as well as the sediment load in the river water, and hence the sediment characteristics of the Fal Estuary creeks.

Particle size is also important in polychaete larval settlement (Hart & Fuller 1979). In the absence of optimum conditions larvae postpone settlement increasing their susceptibility to predation. A change in the sediment grain size of the Fal will have influenced the fauna typically by species replacement, as observed in the Fal, than by changes in the proportions of major taxa present (Warwick 1988).

Observations of and comments on the temporal change in community structure

Perryman (1992) and Somerfield *et al.* (1994b) found that changes from November 1991 to March 1992 were slight and they speculated that these slight changes were due to seasonal effects rather than as a result of the spill. There was no unequivocal evidence to suggest that the Wheal Jane spill had any effects on the macrofauna of the five creeks in their short study over four months. Monitoring data by the National Rivers Authority show that since the spill, heavy metal levels have remained consistent (pers. comm.). Perryman (1992) concluded that the sites should be visited subsequently to their study, in November 1992, to help eliminate the influence of effects soley attributable to seasonality. In reply, this study has shown a change in the communities present in all creeks over the three years. Examination of the three year MDS plots show that the fauna in St Just and Mylor in 1992 have become more similar to the clean control site of Percuil of 1991 indicating that a long-term recovery effect may be occurring, but this is speculative.

Species patterns.

Some species disappeared (*Paranais littoralis, Ampharete acutifrons*) and others appeared (*Phyllodoce mucosa, Heteromastus filiformis*) over the years. These species were all characterised by low numbers. In benthic surveys most attention is traditionally given to the numerically dominant species while rare species are usually considered of minor interest because they contribute little to the total abundance and biomass and are considered as mere random members of communities (Olsgard 1993). This present study indicates that investigation of rare species may be of interest in studies of changes in the benthic community, with only four key species being numerically abundant throughout. Ranking of the abundance of species shows *Nereis diversicolor* and *Nephtys hombergi* to be most

dominant in Restronguet, with *Tubificoides benedeni* and *Streblospio shrubsolii* in the other creeks. The lack of juvenile molluscs in the first year may indicate a disturbance to recruitment in 1990 perhaps related to the tin mine spill.

Contaminant-induced effects?

Is the observed change in faunal composition a result of the spill? A disturbance in benthic communities normally instigates a change in number of species and individuals which result in a change in diversity (Gray 1979). A typical community response to stress after a short period of perturbation, is a reduction in number of species, an increase in total abundance due to an increase of opportunistic species and a corresponding reduction in diversity (Pearson & Rosenberg 1978). In the Fal Estuary there was an increase in number of species and abundance (with the exception of Pill creek), with diversity indices showing no significant changes.

Rygg (1986) studied benthic faunal response to copper, lead and zinc contamination in Norway and described a similar situation to the third year of this study, observing a reduction in numbers of species and abundance. Olsgard (1993) has suggested that this drop in both numbers of species and individuals when exposed to heavy metals is a typical community response for exposure to toxic agents. In the Fal there was a change in number of species and individuals at several sites. In Pill there was a significant reduction in both number of species and abundance compared to the first year. At the other stations there was an increase in species and abundance. This supports the supposition that the classical response model of a benthic community exposed to pollution, with a reduction in number of species and an increase in opportunistic species originally described from studies of organic pollution, is not appropriate when benthic fauna is exposed to toxic substances.

Natural changes?

Various factors might have an important overlying effect on long-term changes in the benthos community, with the main factors being food input, temperature and predation. Buchanan *et al.* (1978) noted that a period of cold winters followed by warmer years resulted in a replacement in the top ranking of some larger species by small species of polychaetes, accompanied by a rise in total numbers of individuals present. Pearson *et al.* (1983) noted similar trends in Loch Linhe and Loch Eil suggesting that climatic fluctuations acted on spawning success and hence subsequent recruitment of particular species. Souprayen *et al.* (1991) further suggested that during cold periods peak productivity of zooplankton and phytoplankton became displaced resulting in an increased supply of carbon to the benthos with a decrease in number of species and increase in abundance and biomass. In this Fal study there was an increase in species one year followed by a decrease the next, except in Percuil, which saw an increase in both species and abundance which could possibly be linked to the recovery after the unusually cold winter of four years ago.

Decreased predation?

Subsequent to the spill of early 1992 there was great local concern expressed for the bird populations of the Fal estuary due to their reliance on sediment dwelling organisms for food. Many observed that the birds appeared sick and sluggish (The Restronguet Society, pers. comm.). If, as a result, their feeding rates or their numbers declined the effects could be an increase in numbers of species or individuals. In particular there was an increase in numbers of juvenile bivalves in the Fal, maybe due to decreased predation. Some of the large polychaetes are also themselves carnivorous predators. Olsgard (1993) found that none of the large predatory macrofauna showed an increase in abundance, and instead noted reduced abundances for most carnivorous species over a two year period. In this

study there were increased rather than reduced numbers of the carnivorous species *Nephtys hombergi*, *Phyllodoce mucosa* and *Glycera capitata* (though the latter two have extremely low abundance). Likewise, there was an increase in deposit feeders and surface deposit feeders in the Fal creeks. Olsgard (1993) also saw a decrease in abundance of deposit feeders.

Overview of the community structure analysis technique - Relative sensitivities of univariate and multivariate techniques

The natural community of animals in the Fal intertidal mud-flats comprise a modest number of species but great abundances of certain species giving a relatively complex set of data. A table of such benthic data is typically complicated to interpret and the techniques described in this chapter have become the routine way of simplification, visual representation and interpretation. They enable characterisation of community attributes, their analysis over spatial and temporal scales, and the establishment of relationships between the observed changes and potentially causal environmental variables. This chapter applied two typical analytical procedures, univariate and multivariate, in an attempt to describe the community present with respect to the heavy metal contaminated estuary over a three year duration.

The study found that multivariate techniques are more sensitive than univariate, as Warwick & Clarke (1991) also found in their comprehensive comparison of techniques which they applied to various data sets such as Ekofisk oil-field macrobenthos and Tamar estuary meiofauna. The sensitivity and generality makes them a particularly valuable tool in the assessment of community change. The BIOENV program effectively showed a relationship between ordinations of sites with respect to their faunistic attributes to environmental data,

and provided a means by which the most important environmental variables responsible for the community differences and change could be identified. These were found to be sediment zinc and copper concentrations.

Clarke and Warwick (1994) questioned whether species dependent multivariate and species independent univariate attributes of community structure behave the same or differently in response to environmental changes, and which is the most sensitive. They concluded firstly, that the similarity in community structure between sites or times based on their univariate attributes is different from their clustering in the multivariate analysis. In the Fal, Restronguet Creek differs from the other four under univariate analysis, but all five differ under the multivariate approach. Therefore, in this study MDS was better at discriminating between stations than the diversity measures. Secondly, the species-dependent multivariate method is sensitive in discriminating temporally (over three years). Thirdly, by matching multivariate ordinations of environmental data to the ordination of faunistic data, the key environmental variables for community change were identified as linked to zinc and copper, and organic matter to a lesser degree i.e. that the community related better to metals than to natural environmental variables (salinity, pH and fines).

However, it is important to apply various data analyses as each will give different information and this will aid interpretation. Sensitive multivariate methods give an early indication that community changes are occurring, but indications that these changes are deleterious are required by environmental managers which is where the less sensitive species-independent diversity measures are applicable. The species independent methods of community analysis, although less sensitive in detecting change than multivariate methods, do have an advantage in that some value judgement can be attached to the changes

observed (Warwick & Clarke 1991). The reduction in species diversity is usually regarded as a detrimental response. However, this is not as simple; Olsgard (1993) has pointed out that metal pollution may have a different effect from that of organic pollution. Also Clarke & Warwick (1994) showed that diversity does not behave consistently or predictably in response to environmental stress; increasing levels of stress and disturbance may either decrease or increase diversity, or it may stay the same. However, the Index of multivariate Dispersion is a multivariate analysis which gives a value judgement, with the higher value being indicative of more stress.

CONCLUSION

Benthic macrofauna community structure analysis has effectively established temporal and spatial differences in the creeks of the Fal estuary. The spatial variation has been linked to heavy metal contamination in sediments rather than to natural variables, shown convincingly by the program BIOENV. There has been a temporal shift in the communities which is not related to changing metal levels which remained constant. Analysis of biotic data at varying levels of taxonomic classification has revealed that little information is lost at higher taxonomic levels. It has been an effective monitoring tool in detection of the heavy metal gradient of the Fal Estuary.

CHAPTER 4

Population Size Structure Studies: *Nephtys hombergi*

INTRODUCTION

General introduction.

The sizes and frequencies of a species are measures that can be used to indicate the condition of a population. They are an integration of the environmental conditions over a time period and therefore can reflect annual growth and reproduction. In this chapter, a **size-frequency (SF) analysis** approach was applied to data from measurements of the polychaete *Nephtys hombergi* to investigate its suitability as a means for estimating the effects of heavy metal contamination at the population level of biological organisation.

Underlying principles.

The hypothesis behind this approach is that naturally occurring processes will affect individuals of a population slightly differently, leading to animals of the same age (class or cohort) having different sizes and variable length life spans.

Throughout various stages of its life history (*e.g.* gonad development, growth, spawning, settlement, etc.), a population may be affected by many factors, both abiotic and biotic. Demographic studies, *i.e.* population dynamics, can investigate independently resultant population characteristics *e.g.* size (Olive 1977, Alvarez-Cadena 1993), recruitment,

growth rate (Williamson & Kendall 1981, Suzuki *et al.* 1986), production (Warwick & Price 1975, Oyenakan 1986), mortality (Saila & Lough 1981) or fecundity (Hakkari & Bagge 1992, Casillas *et al.* 1991, Rundle 1993). However, it is impractical to look at them all separately and instead, an examination of the **age / size frequency structure** can provide an overall indication of the health, condition or performance of a population (*e.g.* Ropes & Merrill 1970, Hartnoll & Bryant 1990, Ebert *et al.* 1993).

Ideally, the species chosen for a SF study should be easy to find and collect in sufficient numbers, have a reasonable literature on its biology to enable interpretation of data, and have a life span appropriate to the study. In addition there is a need to be able to determine the age of organisms. However, age determination can be a fundamental problem when studying marine invertebrates (Olive & Morgan 1983), because it is not usually possible to allocate a precise age to specific animals on the basis of size alone.

Data are typically presented as SF histograms and, in most cases, each peak on a graph represents an age class. Interpretation of SF histograms can indicate growth (by differences in the distances between peaks), recruitment (by comparison of the amplitude of the first ('0') age class), and mortality or emigration (by the annual decrease of amplitude of a class peak). Comparison of SF data can be applied temporally or, as in this study, spatially, providing an indication of the performance of a species at different locations, and can be used retrospectively to make broad conclusions about past conditions from a single sample.

Methods of age determination by size structure analysis.

Direct observation of individuals.

Growth marks

Some studies have used marine organisms whose hard parts which show distinct striations resulting from an alteration of the growth rate associated with spawning or seasonal environmental fluctuations (Olive 1979, Williamson & Kendall 1981). However, such overt growth marks are found only in a minority of species. Examples include rings on fish scales or otoliths, or growth marks on crustacean and mollusc shells, or on polychaete jaws *e.g.* growth lines in the jaws of Nephtyidae used as a means of ageing by Kirkegaard (1970, 1978) and Olive (1977) who assumed that they were laid down annually, but this has not been critically tested (Olive & Morgan 1983). However, these marks can be caused by other events that affect growth such as disease, habitat disturbance or stress due to handling, *i.e.* they are not unequivocally laid down annually. Also, as animals get older, they become more difficult to age with confidence because their reduced linear growth makes it harder to identify their growth bands (Crisp 1984).

Tagging

Tagging or marking of individuals has been used in studies by applying coloured paint to, for example, the gastropod *Monodonta lineata* (Williamson & Kendall 1981). However, this could make the organism more conspicuous and so liable to predation which would affect the ultimate sample composition.

Inference from population structure.

The above two methods are not suitable for all species. An alternative is to estimate population parameters directly from population size data, *i.e.* SF analysis. The approach assumes that recruitment to the population occurs in discrete pulses resulting in cohorts which can be distinguished graphically, statistically or mathematically. The length, weight or width of individuals of a population can be expressed by plotting histograms of the number of individuals in regular size classes (*i.e.* size-frequency histograms), from which year classes (cohorts) can be identified.

The method is particularly applicable to species of temperate marine invertebrates which normally reproduce once a year (Olive & Morgan 1983, Grant *et al.* 1987, Ebert *et al.* 1993. Annual breeding enables the age of individuals to be estimated by their size provided that the annual 'cohorts' do not fully overlap. In a species with a discrete reproductive period, a size frequency histogram will normally have clear modes or peaks representing recruitment for the first one or two years as the youngest generations are usually clearly separated. In later years there is often diminishment in linear growth which can lead to an overlap of year classes. Overlapping can also result from prolonged breeding seasons or extreme variation in individual growth.

Petersen (1891) provided the first and simplest method of separating the age classes in which each of the modes on a size-frequency histogram was taken to be a single class, and all the age classes were assumed present. This allowed the size at each age or the age at each size to be determined. In clear graphs, the position of the mode or peak along the histogram axis gave an approximate measure of the mean size of each year class, and the difference between the two modes indicated the yearly growth rate.

Other graphical methods of analysis were subsequently proposed by Harding (1949), Cassie (1954) and Bhattacharya (1967). Grant (1989), however, reanalysed two classical examples of Harding (1949) & Cassie (1954) and illustrated how interpretation of their data could give misleading conclusions. He concluded that cohorts must be well separated and sample sizes large enough to show clear modes on a size frequency histogram for reliable results from graphical methods. Cohen (1966) proposed that samples of 1000 or more are required, while Grant *et al.* (1987) demonstrated that samples of 500 could not be reliably segregated into cohorts unless each group is separated by three standard deviations. Normally, sample sizes far smaller than these have been used (*e.g.* Warwick & Price 1975, Alvarez-Cadena 1993). A further restriction often overlooked is the assumption that populations are mixtures of normal distributions with no missing age classes, but this may not always be so (Grant *et al.* 1987).

Subsequent mathematical procedures for analysis such as that of MacDonald & Pitcher (1979) can optimise the fit of distribution curves reducing the subjective element of graphical methods. Fisheries research workers have modelled size distributions to gain insight into the underlying processes that give rise to observed patterns (Barry & Tegner 1990, Hartnoll & Bryant 1990). A Ford-Walford plot (cumulative length against year/rings) can be constructed using data from a few shells, for instance, with clear growth rings. This plot can be used to determine the size limits of cohorts, enabling the size-frequency histograms to be divided accordingly into year classes. However, it may not be clear as to how representative those few individuals are of the total population.

Previous size frequency studies

For many organisms, size data are easy to gather. Warwick (1983) reviewed examples of size-frequency distributions for various species and situations. However, there are few studies relating size-frequency to pollution effects, most work having been done on natural environmental effects on growth and survival. Olive (1977) described the size of different age-groups of *Nephtys hombergi* in the Tyne estuary, Kendall (1987a) investigated variation in recruitment and population structure of *Monodonta lineata* and *Gibbula umbilicalis* in Wales, Oyenakan (1986) studied sublittoral *N. hombergi* population dynamics in Southampton Water, Alvarez-Cadena (1993) studied the chaetognath *Sagitta elegans* in the Irish Sea, while Suzuki *et al.* (1996) used the SF techniques to estimate growth of the whelk *Neptunea arthritica* in Japan for fisheries management. SFs have also been used in production estimates *e.g.* Buchanan & Warwick (1974) used multiple size frequency histograms presented in a temporal sequence in their study of macrofaunal production off the Northumberland coast and the annual production of *Donax vittatus* in Carmarthen Bay.

Test organism: *Nephtys hombergi*

Nephtys hombergi (Polychaeta: Nephtyidae) is widely distributed in north-west Europe. It inhabits intertidal and sublittoral sand, muddy sand, mud and gravel. Due to the lack of sediment in the gut on dissection it has been suggested (*e.g.* Fish & Fish 1989) that they are solely carnivorous, feeding on molluscs, crustaceans and other polychaetes. However, Warwick & Price (1975) argued that such a dominant species of mud flats could not be an exclusive carnivore and must also feed on meiofauna and microfauna, or on animals that display tidal migrations not found in intertidal areas. The larvae are said to be pelagic and adults can live up to 6 years (Kirkegaard 1978, Olive 1985, Fish & Fish 1989).

As in all invertebrates with high reproductive capacity, the mortality rate of *N. hombergi* is high, particularly in the larval and juvenile stages. Kirkegaard (1978) found that pre-settlement mortality of *N. hombergi* in Danish waters was *c.* 97%. Post-settlement mortality of *N. hombergi* in the Wadden Sea during a summer was 90%. Although trivial in terms of only one female needing to produce two offspring to maintain population stability, this has important implications in terms of understanding SF distributions. High juvenile mortality is typically expressed on SFs by a large first year I cohort and a reduced year II cohort.

Reproductive failure has been observed in some years in *N. hombergi*. In some instances gravid females failed to discharge their gametocytes (Olive 1985), resorbing them instead. In other cases, the gametocytes produced were not viable, having grossly abnormal cytology (Olive 1983).

There is conflicting evidence for spawning of *N. hombergi*. On the basis of endocrinology and histology, rather than recruitment, *N. hombergi* spawns once a year, but at slightly different times of the year in different areas *e.g.* in the Limfjord *N. hombergi* propagates during the summer and in the Tyne Estuary in May (Olive 1983). In contrast, based on recruitment, Oyenakan (1986) found that it spawned throughout the year with peaks in July and November. However, with respect to this last report, there has been controversy as to whether it refers to true *N. hombergi* or to the closely related, mainly offshore species, *N. kirsivalensis* or *N. assimilis*, as observations of two recruitment peaks may suggest a confusion of the two species (M. Kendall pers. comm.). As a general rule the true *N. hombergi*, as considered in this thesis, are intertidal and *N. kirsivalensis* and *N. assimilis* are offshore.

Aims

This study aimed to relate spatial variation in *Nephtys hombergi* to patterns of heavy metal concentrations in sediments, using size-frequency distribution to see if comparing (a) the abundance, and (b) the sizes of different age classes of *N. hombergi* between different locations would reflect the known heavy metal gradient, the hypothesis being that an impacted site might have smaller mean year classes and fewer individuals per year class.

MATERIALS AND METHODS

Species choice

The general community faunistic survey (see Chapter 3) formed a basis for the selection of the 'key' species to be used for this part of the investigation. *Tubificoides benedeni, Streblospio shrubsolii* and *Nephtys hombergi* were the numerically dominant species in the surveys, whilst *Nereis diversicolor* was irregularly present in creek sites, and bivalves such as *Scrobicularia plana* were generally present only as small juveniles. Polychaetes such as *T. benedeni* and *S. shrubsolii* are inconvenient to work with due to their small size and short life spans. Hence *N. hombergi* was the most practical species to use.

Sampling

Specimens were collected using a box core (50 x 50 x 10 cm or 25,000 cm^3) in March 1994 from mid-intertidal locations (see Chapter 2). On the basis of earlier work (Williams 1994), it was estimated that five cores (0.125 m^3) per creek would provide adequate numbers of *N. hombergi* for this part of the study. After sampling, the sediment was washed through a sieve with 250 µm mesh width, firstly *in situ* to remove the majority of sediment to facilitate transportation and again more thoroughly in the laboratory. The samples were fixed with 10% buffered formalin, stained with Rose Bengal and stored in 70% alcohol. Larger specimens were sorted by eye and smaller ones under a binocular microscope.

Measurements

Because many specimens had lost their tail ends during the sieving process, measurements of the width of the first segment behind the head were made instead of the overall length. Each specimen was removed from the alcohol and blotted with filter paper immediately and measured to the nearest 0.1 mm using callipers and a graticule under a binocular microscope. Jaws of some specimens were examined under a high power microscope for growth rings (Kirkegaard 1970) but results proved inconclusive as the bands were indistinct and could not be distinguished with confidence. Image analysis was attempted to provide more rapid measurements of *N. hombergi* specimens, but the results were unreliable due to the inadequacies of the computer programme available. However, this could be developed and improved in a future study.

These were constructed by grouping individuals by size at 1 mm intervals and then analysing the data graphically to determine age classes. This simple method of size-frequency analysis was possible because the histograms had relatively clear peaks and supportive information was available from other estuaries in the area (J. Davey pers. comm.). Lines of separation were drawn perpendicular to the size axis at the points where the component cohorts apparently intersected, thus segregating groups of individuals into their approximate age classes.

RESULTS

Results are summarised in Table 4.1 and illustrated by the size frequency histograms in Figure 4.1. The SF histograms are divided into year classes (cohorts), and can be examined with respect to their abundance and year class characteristics.

Creek	Class	Min width	Max width	Mean width	Growth Rate	Numbers
Restrong.	0	-	-	-	-	0
	I	0.6	1.1	0.81 ± 0.14	-	14
	II	1.2	2.2	1.6 ± 0.22	1.5	29
	III+	2.3	3.7	2.7 ± 0.41	-	13
						56
Mylor	0	-	-	-	-	0
	I	0.4	1.1	0.81 ± 0.18	-	15
	II	1.2	2.2	1.6 ± 0.23	1.5	66
	III+	2.3	4.0	2.8 ± 0.54	-	14
						95
Pill	0	0.2	0.2	0.2 ± 0.00	-	2
	I	0.6	1.4	1.1 ± 0.18	0.9	25
	II	1.5	2.4	1.9 ± 0.21	0.8	37
	III+	2.6	4.4	3.4 ± 0.80	-	4
						68
St Just	0	-	-	-	-	0
	I	1.1	1.4	1.2 ± 0.12	-	8
	II	1.6	1.9	1.8 ± 0.14	0.6	7
	III+	2.3	3.9	3.3± 0.44	1.5	20
						35
Percuil	0	0.1	0.2	0.16 ± 0.05	-	3
	I	0.4	1.1	0.74 ± 0.21	0.6	68
	II	1.2	1.7	1.4 ± 0.14	0.7	35
	III	1.8	2.2	2.0 ± 0.12	0.6	15
	IV+	2.3	3.5	2.7 ± 0.32	0.7	32
						153

Table 4.1 Summary of *Nephtys hombergi* measurements (mm): maximum, minimum, mean widths (± S.D.), growth rates and numbers of individuals per class and per total sample.

Abundance

The number of *N. hombergi* retrieved from the core samples, *i.e.* the abundance, was unexpectedly low and hence the SF are difficult to interpret with confidence, although relatively clear separation of cohorts was apparent, apart from Percuil samples where there was some overlap. Besides being low, the total abundance in 0.125 m^3 sediment varied significantly ($p < 5\%$) between creeks. St. Just creek samples had the smallest numbers (35 per 0.125 m^3) and Percuil the greatest (153 per 0.125 m^3). Restronguet and Pill Creeks did not differ significantly. The ordering of density of individuals per total sediment sample from each creek (Percuil > Mylor > Pill > Restronguet > St. Just) did not correspond with the order of the heavy metal gradient.

Sizes and year classes

The size-frequency plots (see Figure 4.1) from the first three creeks are tri-modal ('0', I and II), the first mode being animals settling that spring or previous autumn, the second (and in most cases the largest of the peaks) consisting of animals 1 year / 18 months old, the third a year older, and the last is a compound of all other year classes.

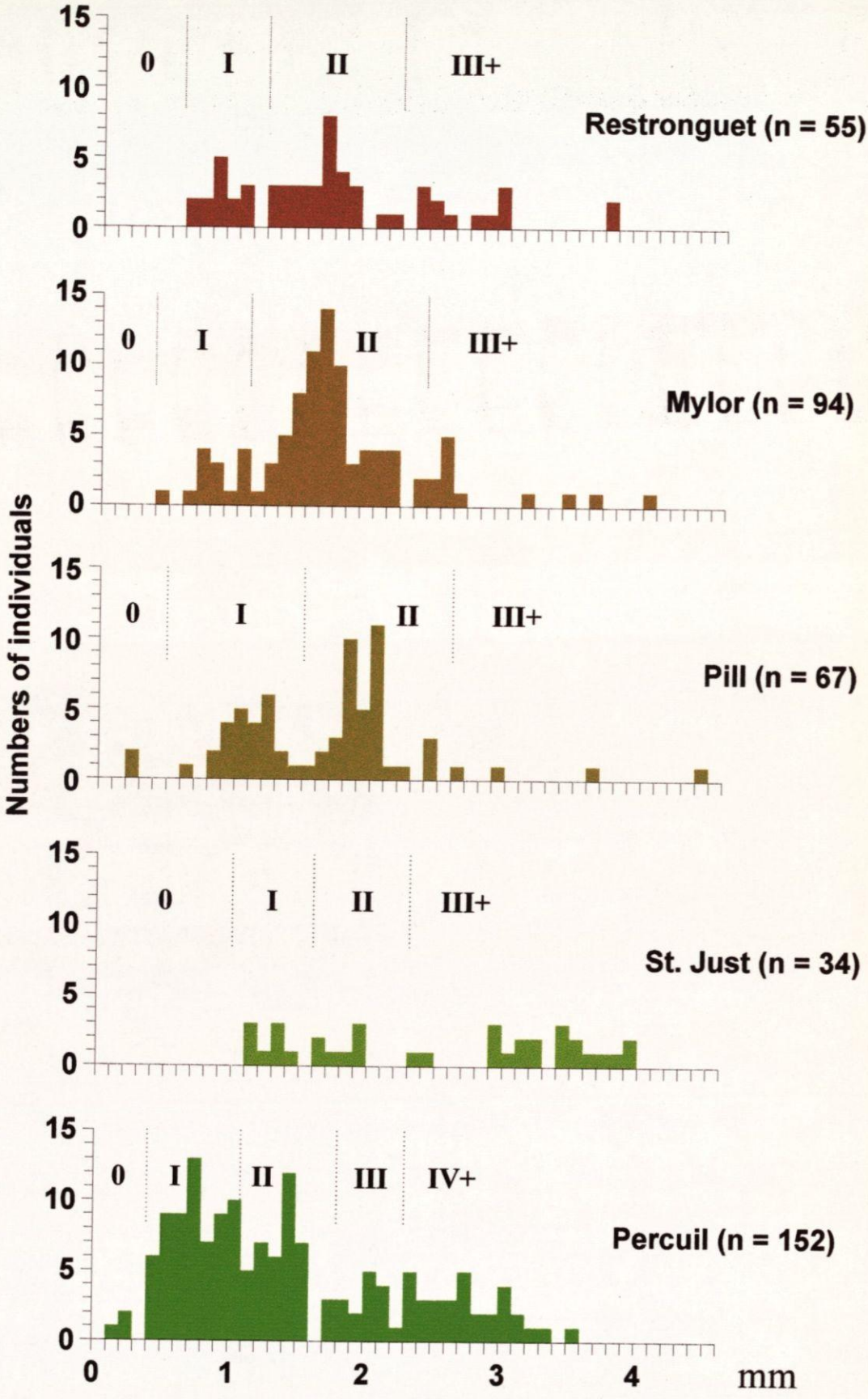


Figure 4.1 Size-frequency histograms for *Nephtys hombergi*

The mean size of individuals did not significantly differ between creeks, with the exception of Percuil Creek with smaller individuals and St. Just Creek larger animals ($p < 1\%$). Patterns again did not correspond with the metal gradient (for statistical comparisons see Chapter 8). Likewise, growth rates, *i.e.* differences between sizes I and II year classes were not significantly different and failed to relate to the metal gradient.

The '0' class was discernible in Pill and Percuil Creek samples but absent from the Restronguet, Mylor and St Just samples. There is a possibility that the smallest animals were not retained on the sieve during sample processing. However, numbers were too small to enable a meaningful interpretation.

The year I cohorts from Percuil Creek were dominant inferring that either the populations were expanding, or that there had been high juvenile mortality, or that there had been predation, or that the '0' class had not recruited. The I and II cohorts were distinct in Restronguet Creek, apparent but slightly overlapping in Mylor and Pill Creeks, too small in St. Just to segregate with confidence, and merged in Percuil Creek samples.

Only the St. Just and Percuil samples showed a strong III - III+ class. The former had an even but weakly represented spread of individuals throughout. Samples from the other creeks were very under represented and contained even fewer numbers preventing determination of a cohort pattern for these older age classes. Thus separation of these groups could only be subjective as the contribution by the larger and slower growing groups was relatively small.

DISCUSSION

Does the size frequency information relate to the heavy metal contamination?

No clear trend that relates to the heavy metal contamination gradient was evident (for statistics see Chapter 8). Contrary to the expectation that higher contamination would lead to reduced scope for growth and hence smaller individuals, the largest animals were in St. Just Creek rather than in Percuil. It could be argued that those animals that survive the conditions in the early months of life have less competition and hence would grow well.

Factors which could affect the growth and size of *Nephtys hombergi*.

If not apparently related to the heavy metal gradient, other influences, both abiotic or biotic might be involved:

Abiotic factors

Salinity, organic matter and food supply.

Natural environmental factors such as salinity, sediment grain size, organic content or tidal exposure, as well as contaminants, can affect size patterns. In estuaries, salinity can be important as, outside the optimal range, animals expend more energy in osmoregulation and consequently have less energy available for growth. Warwick (1983) investigated salinity effects in the Severn Estuary where the annual growth was much greater in the middle salinity range than at either low or high salinity. However, in the Fal Estuary salinity was not significantly different between creeks (see Chapter 2) and hence is apparently not an influential factor in this study although temporal variation was not been measured.

Kirkegaard (1978) noted that animals living in mud with high organic content in experimental tanks showed a greater growth rate than those with a low organic content. In the Fal Estuary, the percentage organic matter was highest in St. Just creek relative to the others in the year of sampling. Later in the study there was a prevalence of the algae *Enteromorpha* on the upper shore regions of St. Just Creek which may have increased organic matter content of the sediments, which in turn may account for the larger specimens of *N. hombergi*, but not for their low abundance.

Growth of *N. hombergi* depends on the food supply and availability of its prey. In his report about the Dogger Bank, Kirkegaard (1978) showed that greater general polychaete density provided a greater food supply which led to the larger growth rate of local *Nephtys* individuals. In this study, the community investigation (see Chapter 3) showed that density of polychaetes was significantly greater in Percuil Creek in the sampling year and this may have provided a greater food supply for carnivorous polychaetes. The consequence of this could be overcrowding leading to competition and thus result in the smaller animals observed in Percuil Creek.

Biotic factors

Density and competition, predation, settling time and recruitment.

Density of individuals can affect size through competition. In Percuil Creek there was a high density of *N. hombergi* relative to the other creeks and correspondingly smaller individuals. This might be related to intraspecific competition for available food and/or space (Kirkegaard 1978). In contrast, there was a low density of individuals in St. Just creek with correspondingly larger individuals.

Predation can influence estimates of growth rate, particularly if predators such as fish, crabs or birds prefer and find larger specimens (Kirkegaard 1978). A possible lack of predators in the contaminated waters of Restronguet, Mylor and Pill Creeks could account for the larger specimens of *Nephtys* present. The Restronguet Society (pers. comm.) reported that birds were affected detrimentally by the polluted waters from the Wheal Jane tin mine spill and this could have affected the numbers of larger *N. hombergi* in Restronguet Creek.

An important factor for the growth of juveniles is their settling time and recruitment, with those settling first expected to become the largest. The small numbers of individuals in St. Just could be due to consistently poor recruitment. Sampling sites in Percuil Creek from which abundance was greatest, were further up the inlet. Thus if pelagic larvae arrive from slightly offshore as suggested by Kirkegaard (1978) for *Nephtys* spp. on the Dogger Bank, they would have further to travel, resulting in later settling and hence smaller specimens. However, it is doubtful that young *Nephtys hombergi* spend their pelagic stage offshore, remaining more local to their site of origin and ultimate settling (Kendall pers. comm.). Had they been recruited from offshore, it is unlikely that they would naturally have the observed heavy metal tolerance (Bryan & Gibbs 1983).

Criticisms of and difficulties with the technique

Substantial effort was involved in obtaining enough specimens for data analysis by comparison with the numbers required for general faunistic surveys. Collection was difficult in the estuarine mud-flats and sample processing was highly labour intensive. The bulky sediment samples required sieving *in situ* but access to a water source was usually difficult. The technique would have benefited from greater numbers of specimens, but even larger samples of sediment would have had to have been collected. A preliminary survey might

have revealed how much more sampling was needed at each site to provide adequate numbers of polychaetes for analysis. Additionally, a separate sampling method for the '0' class (*e.g.* as used by Olive & Morgan (1983)) would be necessary to enable completion of the analysis.

Analytical techniques for comparison of size frequency plots do have a subjective element, using visual inspection and identification. Although this study was based on small samples it was usually not difficult to define classes I and II using the graphical technique, but the older age classes were poorly represented and defined.

Overall, the technique is difficult to interpret, especially as animals retained on the 250 μm sieve may not be truly representative of those in the sediment, with younger individuals not being retained. Interpretation is particularly difficult when age classes are absent although generally, where all sites in one area suffer, some broad-scale factor(s) (*e.g.* weather) may be responsible. But if a local site suffers a decline it could signify something more specific such as pollution, although attributing blame can be difficult. The reason for the extremely small abundance of '0' classes from all creeks in this study was not obvious and could benefit from a much more detailed investigation.

CONCLUSION

In conclusion, there was no apparent relationship between the size frequency distributions of *Nephtys hombergi* with the heavy metal gradient of the Fal Estuary creeks. As a pollution monitoring tool the method was time-consuming and labour intensive with comparison of size frequency distributions relying on subjective assessments, reducing the confidence in the interpretation of the results. Whilst sample collection was the biggest obstacle of the technique, measurement of individual specimens presented difficulties. Damage to specimens meant that physical measurements had to be made of the width of the first segment rather than the overall length which would have been easier. An image analysis technique could have simplified this part of the procedure but although a machine was available, it proved unreliable and it was beyond the scope of this investigation to develop it further.

CHAPTER 5

Scope For Growth: Physiological Energetics Of *Mytilus edulis*

INTRODUCTION

Measurement of the energy available for growth, termed "scope for growth" (SfG) (Warren & Davies 1967), provides a quantitative assessment of the energy status of an animal as well as an important indication of the individual components (and hence mechanisms of toxicity) which affect changes in growth rate (Widdows 1985). SfG is determined by measuring physiological traits (rates of feeding, digestion, respiration and excretion), and integrating the results using physiological energetics via a balanced energy equation. It provides an insight into the energy available for growth and reproduction and how these might be disrupted by environmental stress and pollution (Bayne & Newell 1983). Evaluation of SfG rather than directly measuring growth itself, has been successful in assessing the biological effects of pollutants (Widdows & Johnson 1988, Widdows *et al.* 1987, Widdows *et al.* 1995). There is also agreement between indirect estimates of growth based in this way on the energy budget and the more direct determinations of biomass production based on detailed population size-class analysis (Gilfillan & Vandermeulan 1978, Bayne & Worral 1980).

Other studies

Locations applied to.

Environmental pollution assessment measuring physiological energy of mussels combined with chemical analyses of contaminants in their tissues, has been successful in many field situations, detecting effects in both temperate and tropical environments (Widdows & Salkeld 1992b). Contamination gradients over 10 km have been detected in Narragansett Bay , USA (Widdows *et al.* 1981), San Francisco Bay, USA (Martin 1985), a sewage dump site in Plymouth, UK (Lack & Johnson 1985), Hamilton Harbour, Bermuda (Widdows *et al.* 1990). A more extensive study covered 1000 km coastline bordering the North Sea (Widdows *et al.* 1995) and showed that SfG reflected overall increases in a suite of environmental contaminants from Scotland to the Thames, with specific sites *e.g.* Humber to the Wash, showing markedly reduced SfG although at no sites did metals accumulate at concentrations that might cause a significant reduction.

Pollutants applied to.

The technique has been applied to various pollutant effects. Previous field and laboratory studies have demonstrated that clearance rate and SfG of *Mytilus edulis* are responsive to organic toxicants including petroleum hydrocarbons (Widdows *et al.* 1985), polychlorinated bi-phenyls (PCB's) (Martin 1985), tributyltin (TBT) and dibutyltin (DBT) (Widdows & Page 1993). Little attention has been paid to effects of heavy metals, though they were included in the Bermuda study but were of insignificant effect relative to the other contaminants (Widdows *et al.* 1995).

A critical level for copper of 59 mg kg^{-1} mussel tissue was ultimately lethal to *M. edulis*, regardless of copper concentration in the water or exposure time (Martin 1979). In contrast to petroleum hydrocarbons with no threshold effect, copper exerts sub-lethal effects on feeding and growth rates of *M. edulis* over a relatively narrow concentration range (less than one order of magnitude) with a threshold effect at around 5 to 10 mg l^{-1} copper and a marked inhibition at 20 mg l^{-1} (Manley *et al.*. 1984, Redpath 1985, Widdows & Johnson 1988). Widdows *et al.* (1984) reported levels of 16 mg g^{-1} lead dry weight in the Tamar estuary, but no evidence that lead has deleterious effects on mussels at these elevated levels. *M. edulis* and its SfG have been used commonly by bodies such as the NRA to monitor marine contamination.

Environmental factors applied to.

Many studies have involved determining SfG in relation to non-pollutant effects such as temperature (Widdows & Bayne 1971), food ration and body size (Thompson & Bayne 1974), and seasonal cycle (Bayne & Widdows 1978).

Other species applied to.

Bakke (1988) used *Littorina littorea* exposed to combined pollutant stress but found that SfG failed to discriminate between treatments and thus saw a need to develop the method for this species. However, greater success has been achieved with *Aulacomya ater*, *Cerastoderma edule, Perna viridis* and *Scrobicularia plana* (Worral *et al.* 1983a & b). The mussel *Arca zebra* was studied with in a Bermuda contamination gradient (Widdows *et al.*. 1990), *Daphnia magna* in a salinity stressed situation (Baillieul *et al.* 1996), and blood cockles (*Anadara granosa*) in an industrial discharge (Din & Ahamad 1995). Despite these

examples, mussels (*M. edulis*) remain the most popular test organisms, because if not present, they can be readily transplanted.

Transplantation studies.

Transplantation techniques have been used in several field studies. Widdows *et al.* (1981) measured the effects of a pollution gradient on the physiological condition of transplanted mussels in Narragansett Bay, USA and Widdows *et al.* (1990) transplanted mussels to investigate a contamination gradient in Bermuda. The procedure does not affect the physiology of the organisms (Widdows & Salkeld 1992b).

Test organism; *Mytilus edulis*

Feeding and energy budgets.

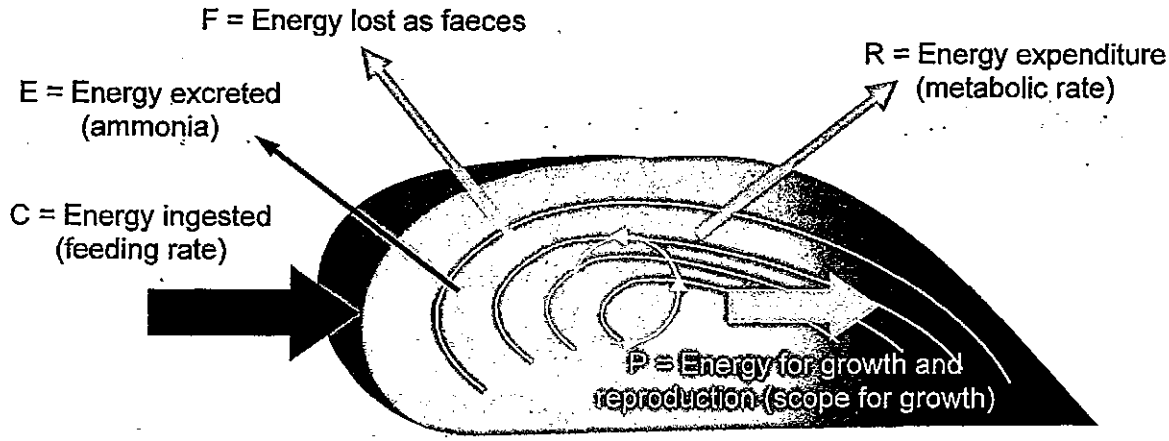
Mussels are suspension feeders filtering particulate organic matter (POM), in the $1\text{-}10^2$ mm size range, by pumping water through their gills. The particles include mostly phytoplankton, but bacteria, protozoan and detritus supplement the algal diet. They can also use dissolved organic matter (DOM) as well as POM. Their feeding structures are ctenidia (gills) with filaments (bearing lateral and frontal cilia, and latero-frontal cirri) and a ventral food groove. Inhalant and exhalant openings or siphons enter the mantle cavity, and labial palps direct food particles to the mouth.

To feed, the shell valves gape, enabling contact between water and the mantle cavity. Beating lateral cilia generate a current of about 1 l h^{-1} that flows between the filaments. Latero-frontal cirri beat to create 1.2 x 4.8 μm meshes between filaments and so filter particles from the current, transferring them to the frontal cilia, which move particles across

the surface of the ctenidia to the food groove. Concentrated within this groove, the particles, at 200,000 x their density in sea water, are moved towards the mouth via the ciliated labial palps which select suitable particles for ingestion and reject unsuitable ones. The latter are bound in mucus as pseudofaeces. Selected particles enter the mouth in suspension and are subsequently subjected to intracellular and extracellular digestion. Undigested material passes through the anus as faeces to join the exhalant current along with pseudo faeces, carbon dioxide, ammonia and, if spawning, gametes.

The feeding rate is measured as filtration or clearance rate (equal to the volume of water cleared of particles per unit time) multiplied by the density of particles. Filtration rate over a time period is influenced by gill area, ciliary beat rate (temperature, salinity, water quality, particle quality and particle concentration effects) and amount of time the shell gapes (water quality, disturbance and tidal height effects). Mussels can influence their food intake rate by controlling shell gaping (via adductor muscle contractions), lateral cilia beat rate, and activity of latero-frontal cirri mechanisms of the labial palps.

The processes (see Figure 5.1) of ingestion (C), assimilation (A) and assimilation efficiency (p), metabolism (M), excretion (U), defecation (F) and growth (G) can be quantified in units of energy (Joules time^{-1}). Growth, as in this investigation, is often expressed as SfG and is calculated from values of P, C, M and U derived from laboratory experiments. Thus factors affecting growth, such as particle concentration, mussel size, temperature, as well as pollutants, can be investigated.



Growth = (Energy gains) - (Energy losses)

P = C - (F + R + E)

Pollution stress causes:- a reduction in growth

Figure 5.1 Diagrammatic representation of the energy budget of a mussel. (Source, Widdows & Salkeld 1992a).

A decline in SfG can occur as a consequence of a reduction in energy intake and / or an increase in energy output (Bayne 1985). A reduction in energy intake may be due to any one or a combination of the following:

1. Reduced feeding rates
2. Reduced food availability
3. Reduced efficiency of digestion and absorption of food material.

An increase in energy output or loss may be caused by:

1. Increased respiration rate
2. Increased excretion rate.

Mussels are capable of maintaining many of their biological processes relatively independent of the normal fluctuations of environmental variables, whilst at the same time remaining responsive to environmental contaminants and natural environmental stressors deviating from the normal range of tolerance (Bayne 1985).

Aims

The purpose of this investigation was to use the procedures of physiological energetics to quantify sub-lethal biological effects along the heavy metal contamination gradient of the Fal estuary with the production of SfG values. The study used the mussel *Mytilus edulis* from transplanted populations as the indicator species.

MATERIALS & METHODS

Sampling

Mussels were from the original batch transplanted to the five selected creeks of the Fal estuary (Figure 2.2, Chapter 2) from the clean site at Bull Hill in the Exe estuary during May 1994. They were sampled from the transplantation cages for testing on the previous day to the experiment, possible because pollutant induced effects are retained after 24 hours (Widdows & Salkeld 1992a). All measurements were made in August 1994, during the summer period of active growth after the spawning season.

Physiological measurements were based on samples of 16 individuals per site. There was a constraint on sample size because of availability of apparatus at Plymouth Marine

Laboratory (PML). Twenty specimens of uniform shell dimensions (4 - 5 cm shell length) were selected (taking care to cut byssus threads rather than wrenching the mussels from their site) and transported in insulated boxes, cooled with freezer packs for the 3 hour journey from the field site to PML. They were cleaned of epibionts, washed, dried and labelled, and maintained over night in static sea water. This allowed the repayment of any oxygen debt from exposure during transportation, the flushing out of ammonia accumulated in the mantle cavity water and tissues, and the resumption of normal filtering (Widdows & Johnson 1988) before physiological measurements the next day.

Physiological recovery is complete within 2 hours of re-immersion following 5 hour air exposure (Widdows & Shick 1985) and within 12 hr after 24 hour exposure at 7°C (Widdows & Salkeld 1992a). It is important that tests are done after the mussels recover from any transportation and handling stress, but before they begin to depurate significant quantities of contaminants and so reduce any pollution induced stress.

Of the twenty specimens, sixteen apparently normally functioning individuals were selected for each experiment. Experiments were conducted using filtered sea-water collected 20 km offshore (Eddystone FSW down to 1 μm). Practical procedures for physiological measurements followed those of Widdows & Salkeld (1992a):-

Feeding rate measurement

This was measured as the 'clearance rate' which is the volume of water cleared of particles per animal $^{-1}$ h^{-1}. It was estimated by measuring the removal of suspended algal particles (*Isochrysis* spp.) from water flowing through experimental chambers containing individual

mussels. All water entering the clearance-rate measuring system was pre-filtered through a 180 μm sieve to remove plankton or other large debris. The apparatus (Figure 5.2) consisted of a variable speed centrifugal pump discharging water into a central mixing chamber (1.5 l volume) with a magnetic stirrer and then through tubing (2.5 mm bore) to 18 chambers in parallel, 16 containing study mussels and two empty as controls. To minimise recirculation of water by the mussel the inflow into each chamber was at the bottom, adjacent to the inhalant mantle edge of the mussels, and the outflow was via an overflow tube at the top. Flow rates through each chamber were constant at approximately 180 ml min^{-1}, estimated to be low enough to record a significant difference between the inflow and outflow particle concentrations, yet sufficient to prevent recirculation of the water by the mussel (Widdows & Johnson 1988). If necessary, particle concentration was adjusted by clamping or declamping the main source tube, modifying the motor speed, then allowing the apparatus to stabilise before checking with an electronic coulter counter particle size analyser to set the required cell concentration (*c.* 7000 cells ml^{-1}).

The clearance rate of each mussel was determined after 60, 120 and 180 minutes. Water samples were collected simultaneously from the outflows of all mussel chambers, by moving a rack of measuring cylinders (200 ml volume) directly beneath them and removing them after 60 seconds. Particle concentrations were measured again by the electronic particle counter using a 140 μm orifice tube. The mean of four counts per sample were taken. Only particles greater than 2 μm were counted in 0.5 ml sub-samples. Water samples from the empty control chambers represented the inflow concentration (C_I) and water sampled from the outflow of each of the sixteen experimental mussels chambers represented

the outflow concentration (C_O). Clearance rate (in l h^{-1}) was calculated as flow rate (in l h^{-1}) multiplied by $(C_1-Co)/C_1$.

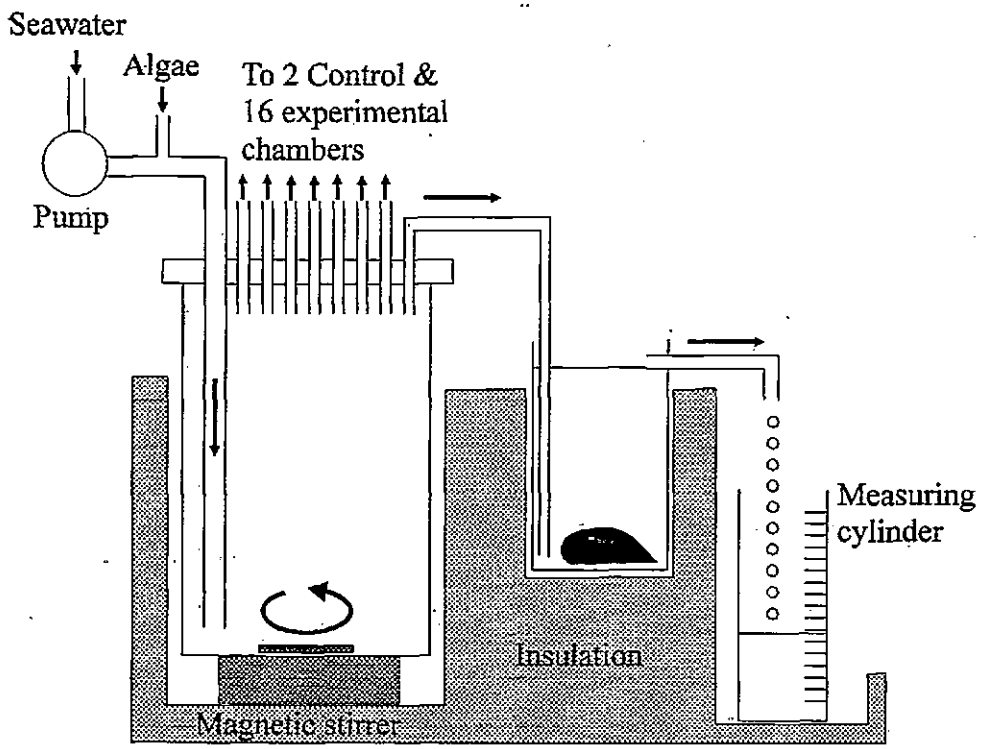


Figure 5.2 Flow-through apparatus for measurement of clearance rate (Source, Widdows & Salkeld 1992a)

Food absorption efficiency.

This represents the efficiency with which organic material is absorbed from the ingested food material or seston. The method used (Conover 1966) is based on the organic fraction in food and faeces and depends upon the assumption that only the organic component of food is significantly affected by the digestive processes. It compares the proportion of organic matter in the food and the faeces.

Conover's ratio = Absorption efficiency = (F - E)/((1 - E)F)

where F = ash-free dry weight : dry weight ratio of food (seston);

E = ash-free dry weight : dry weight ratio of the faeces.

Faeces were collected after mussels have been held for 24 hours in the laboratory at a constant cell concentration, thus allowing the gut contents reflecting their previous diet to be evacuated and discarded. Seston concentration (mg l^{-1}) in the in-flowing water was sampled at about 3 hourly intervals, three replicates at each. Faeces were collected by pipette from the mussel chambers and filtered through washed, ashed and pre-weighed glass fibre filters (4.7 cm, GFC) and the salts removed by washing the filter and its edges with distilled water (3 x 10 ml). Filters were dried at 90°C and weighed to calculate the total dry weight of particulate matter, or seston, per litre of water. They were then ashed at 450°C overnight in a muffle furnace and weighed again in order to calculate the weight of organic material combusted. This component represents the weight of POM or ash-free material. GFC filters were handled only with forceps and stored in desiccators to reduce weight change errors. Blank GFC filters were weighed with each batch of filters to correct for weight change due to atmospheric humidity changes (Widdows & Johnson 1988). Faeces from paired individuals were pooled due to small amounts produced. Absorption efficiency estimation on single individuals is feasible but would require use of micro-calorimetry for determination of organic content by micro balance or elemental carbon analysis (Bakke 1988).

Respiration rate

This was measured as rate of oxygen consumption for the 16 individual mussels in two batches of eight simultaneously in 8 closed glass respirometers (500 ml volume) (Figure 5.3). Air-saturated sea water was carefully poured into each respirometer and kept aerated by a magnetic stirrer bar beneath a perforated glass plate supporting a mussel. The mussels were left for 30 minutes to open their valves and resume normal pumping, before oxygen

uptake was measured over the next hour. The rate of decline in oxygen partial pressure (pO_2) in each chamber was continuously measured by a radiometer oxygen electrode connected to a Strathkelvin oxygen meter. Each of the eight oxygen meters was coupled to a multi-channel chart recorder. The rate of oxygen consumption (V_{O2} ml O_2 h^{-1}) was calculated as follows

$$V_{O2} = 60(C(t_0) - C(t_1))(V_r - V_a)/(t_1 - t_0)$$

where t_0, t_1 = start and finish times (mins) of the measurement period;

$C(t)$ = concentration of oxygen in the water (ml O_2 l^{-1}) at time t;

V_r = volume of the respirometer;

V_a = volume of the mussel.

Oxygen solubility tables were used to convert pO_2 values to oxygen concentration in ml O_2 l^{-1}.

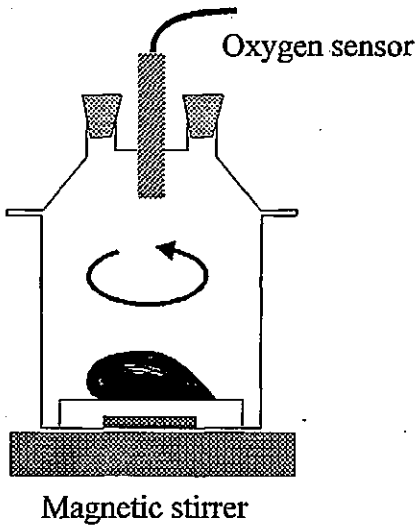


Figure 5.3. Glass respirometer for measurement of oxygen consumption

Ammonia excretion

The rate of ammonia excretion is generally omitted (Widdows & Salkeld 1992a) from physiological energetics measurements, and hence in the calculation of SfG, but it is closely associated with the respiration rate and forms a relatively small proportion (< 5%) of the metabolic energy expenditure.

After all physiological measurements have been completed the shell length and dry tissue weight of each mussel were measured and recorded. Flesh was dissected from the shell and dried to constant weight at 90^{o}C.

Calculation of scope for growth

The energy available for growth and gamete production (SfG) was calculated for each individual. Physiological rates were corrected to a standard body size (1 g dry weight) and then converted into energy equivalents (J h^{-1}) and used in the balanced energy equation to calculate the energy available for SfG, *i.e.* the difference between the energy absorbed from food minus the energy expenditure associated with respiration, using a spreadsheet programme.

1) Energy consumed (C)

$$C = \text{clearance rate } (l\ g^{-1}\ h^{-1}) \times (mg\ l^{-1}\ POM) \times (23\ J\ mg^{-1}\ POM)$$

where the energy content of POM or algal food is *c.* 23 J mg^{-1} ash-free dry weight (Slobodkin & Richman 1961, Widdows *et al.* 1989, Widdows & Salkeld 1992a).

2) Energy absorbed (A)

$$A = (C) \times \text{absorption efficiency}$$

3) Energy respired (R)

$$R = (\text{mmoles } O_2\ g^{-1}\ h^{-1}) \times 0.456$$

where the heat equivalent of oxygen uptake is 0.456 J mmole $^{-1}$ O_2

4) Energy excreted (U)

$$U = (\text{mmoles } NH_4\text{-N}\ h^{-1}) \times 0.349$$

where the excretion of 1 mmole -N h^{-1} is equivalent to an energy loss of 0.349 J h^{-1} (However, this value is zero for this study).

5) SfG (P)

$$P = A - (R + U)$$

i.e. production, P, is estimated from the difference between energy absorbed from the food and the energy expenditure via respiration and excretion, and this is referred to as SfG.

Body condition index (BCI)

BCI = 100 (dry tissue weight) / (shell weight)

This is the proportion of the internal shell volume occupied by the body tissues (Baird 1958 cited in Bayne 1985). An increase in BCI reflects an increase in the organic constituents associated with growth and depends on the balance between food availability, rates of feeding and rates of catabolism. A reduction in the index therefore reflects either periods of stress involving use of reserves or spawning.

Statistical analysis

Statistical differences in food consumption, respiration, SfG, and the ratio of tissue dry weight to shell length were tested by the same programme.

RESULTS

Food uptake and respiration

Physiological responses of *M. edulis* collected from the Fal estuary creeks and the Exe control site are shown in Table 5.1. Clearance rates of mussels from the two most polluted creeks, Restronguet and Mylor, are the greatest and are not significantly different from those from the clean control site, nor do they differ significantly from one another. Clearance rates of mussels from the three least contaminated sites are the smallest and are significantly different ($p < 5\%$) from the control and from the two most polluted sites, but are not significantly different themselves. The rates did not correlate with the heavy metal content of mussels or sediment (see Chapter 8). The pattern is illustrated in Figure 5.4a.

Respiration rates increased proportionally to increasing metal concentration. Rates of oxygen consumption by mussels from the two most polluted sites were not significantly different and were higher than that of the control mussels. Rates of oxygen consumption from the three least polluted sites were significantly different, were inversely proportional to heavy metal levels and significantly lower than the control mussels. Data are illustrated by Figure 5.4b.

Site	Max clearance rate ($l\ h^{-1}$)	Mean clearance rate ($l\ g^{-1}\ h^{-1}$)	Respiration rate ($ml\ O_2\ g^{-1}\ h^{-1}$)
Restronguet	2.1 ± 0.21	3.1 ± 0.27	19.5 ± 1.15
Mylor	3.1 ± 0.23	4.2 ± 0.30	19.3 ± 0.69
Pill	2.5 ± 0.12	2.6 ± 0.14	15.9 ± 0.97
St. Just	2.7 ± 0.15	2.9 ± 0.12	14.9 ± 0.60
Percuil	2.0 ± 0.13	1.8 ± 0.12	13.6 ± 0.86
Exe control	2.9 ± 0.15	3.4 ± 0.18	16.2 ± 0.75

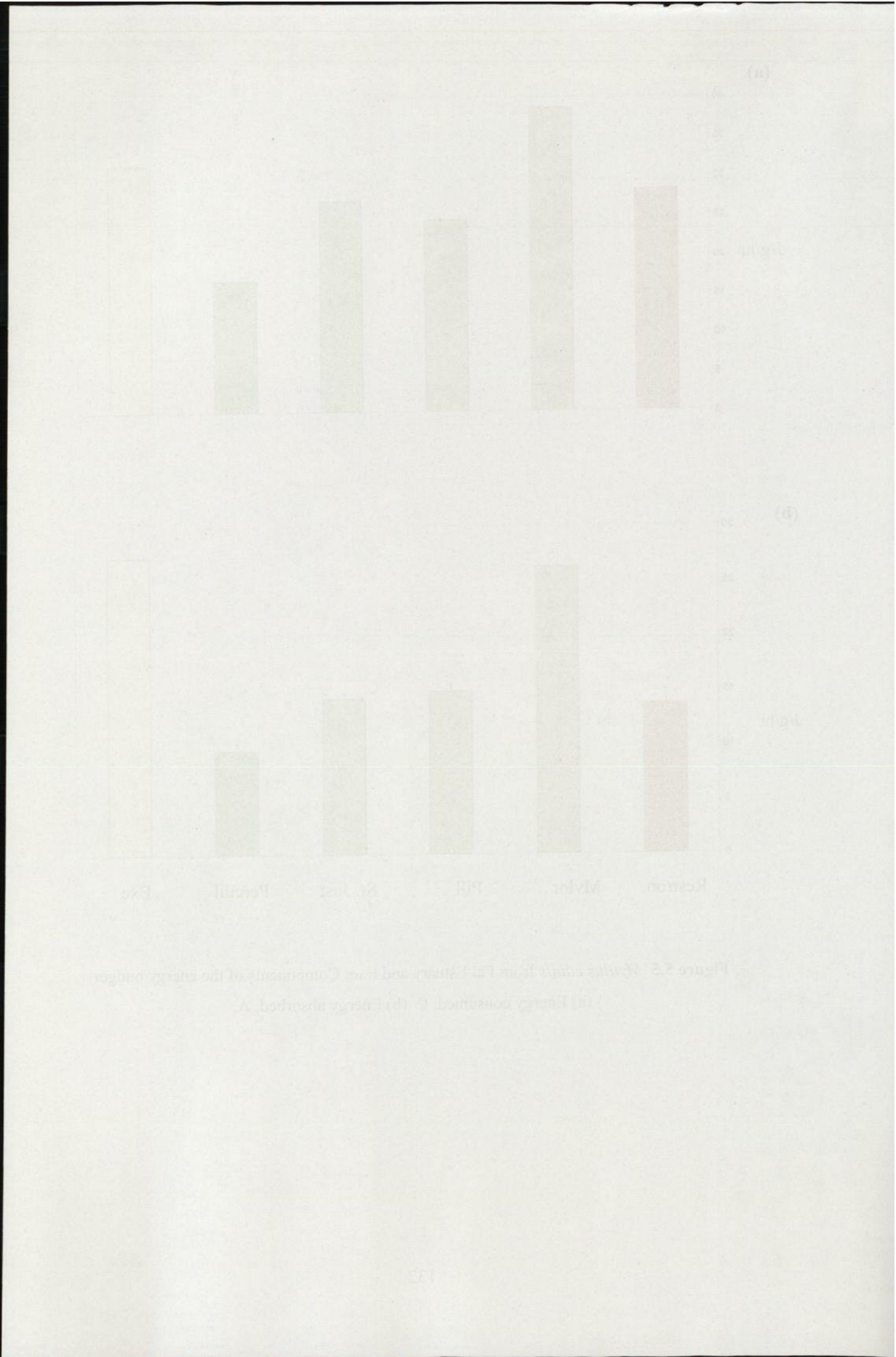
Table 5.1 Physiological responses in mussels (*M. edulis*) collected from Fal Estuary creeks and the control site in the Exe Estuary (mean ± S.E., n = 16).

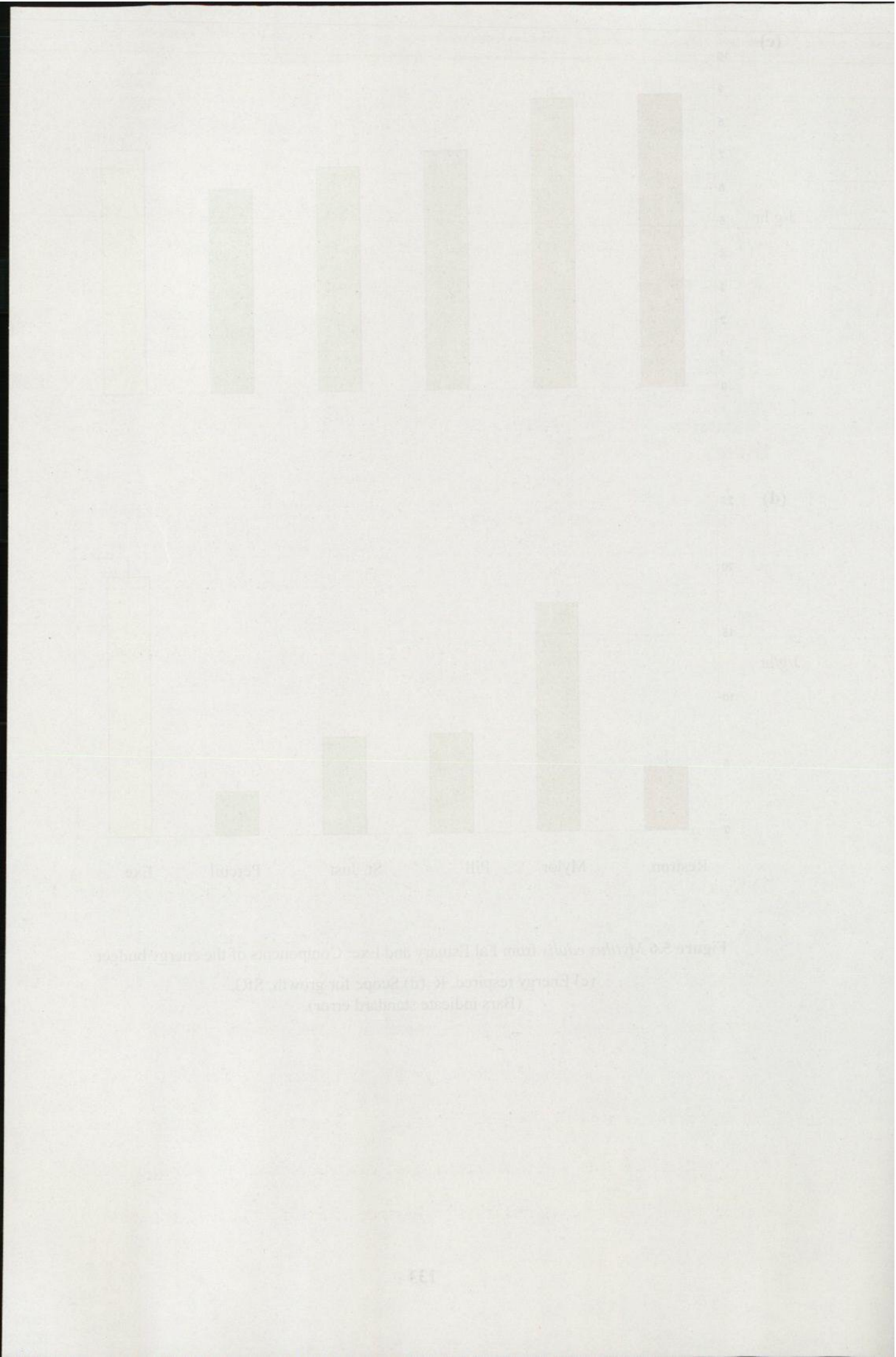
Energy equivalents and SfG

Table 5.2 lists energy equivalents of the physiological responses including SfG values and Figure 5.5 illustrates the data graphically. Values are erratic; mussels from the second most contaminated creek, Mylor, had the highest SfG, but this was not significantly different from the control mussels. The most polluted creek, Restronguet, and the least polluted creek, Percuil, had the lowest SfG, not significantly different from one another. Mussels from the two intermediate contamination sites had intermediate SfG values, significantly different ($p < 5\%$) from all other sites. Rates of SfG are most closely correlated to energy consumed and absorption efficiency. Energy respired was proportional to heavy metal contamination. The ultimate SfG values were not correlated with heavy metal contamination (Chapter 8).

Site	Energy consumed, C	Energy absorbed, A	Energy respired, R	Scope for growth, SfG
Restronguet	28.2 ± 2.5	13.7 ± 1.2	8.8 ± 0.52	4.8 ± 1.2
Mylor	38.6 ± 2.7	26.2 ± 1.8	8.7 ± 0.31	17.4 ± 1.7
Pill	24.4 ± 1.3	14.8 ± 0.7	7.2 ± 0.44	7.6 ± 0.7
St. Just	26.9 ± 1.1	14.2 ± 0.5	6.8 ± 0.27	7.4 ± 0.5
Percuil	16.9 ± 1.1	9.5 ± 0.6	6.2 ± 0.39	3.3 ± 0.8
Exe control	31.4 ± 1.6	27.2 ± 1.4	7.3 ± 0.34	19.8 ± 1.2

Table 5.2. Components of the energy budget and SfG ($J\ g^{-1}\ h^{-1}$) in mussels (*M. edulis*) collected from the Fal Estuary creeks and the control site in the Exe Estuary (mean ± S.E., n = 16).





Shell length and body size / condition

By contrast, Table 5.3 shows that the tissue dry weight and BCI of mussels from the five creeks did follow the pattern of heavy metal contamination with specimens from the most polluted creeks having the smallest body tissue weight (0.57g). Shell lengths did not differ significantly one to another.

Site	Shell length (mm)	Tissue dry wt. (g)	Condition index
Restronguet	47.3 ± 0.20	0.57 ± 0.041	7.790 ± 0.34
Mylor	47.1 ± 0.21	0.63 ± 0.026	8.910 ± 0.42
Pill	47.0 ± 0.26	0.93 ± 0.032	13.353 ± 0.78
St. Just	47.3 ± 0.34	0.89 ± 0.029	12.204 ± 0.82
Percuil	47.2 ± 0.18	1.15 ± 0.043	16.017 ± 0.70
Exe control	47.3 ± 0.28	0.80 ± 0.026	10.953 ± 0.86

Table 5.3 Shell length, tissue weight and body condition index of mussels (*M. edulis*) collected from the Fal Estuary and the Exe Estuary control site (mean ± S.E., n = 16).

DISCUSSION

Summary

The purpose was to evaluate how the energy condition of mussels from transplanted populations reflected the persistent pollution gradient of the Fal estuary. *M. edulis* from the five Fal estuary creeks did not show a direct relationship between SfG and the gradient of heavy metal contamination. Although all creeks, with the exception of Mylor the second most polluted creek, had significantly lower SfG than the control site mussels indicating that they were stressed by being in the Fal estuary environment. Measurement of the major components of the energy budget demonstrated that the variation in growth potential of mussels was primarily due to reduction in clearance or feeding rate, as previous literature

also found (Widdows & Johnson 1988, Bakke 1988). Surprisingly, Mylor was apparently unstressed, exhibiting a more efficient food absorption rate and higher SfG than the control mussels, again linked to feeding rate. Perhaps some source of ample food supply compensated for any energetic disadvantage due to chemical contamination, but this is not reflected in their body size. The individual component of respiration rate, as well as body size and condition did reflect the heavy metal gradient and discriminated between sites. BCI for the three least polluted sites was greater than for the original control population. The advantage of this latter index is that it is a simple measurement to make requiring only basic laboratory equipment; the disadvantages are reported to be its insensitivity and its low signal-to-noise ratio, but it gave relatively accurate results in this current study.

Previous studies.

SfG values of -5 to 5 J $g^{-1}h^{-1}$ indicate high stress, 5 to 15 indicates moderate stress, and 15 to 25 J $g^{-1}h^{-1}$ indicates low stress (Widdows & Salkeld 1992a). In comparison with other sites, mussels in the Fal estuary experience severe levels of heavy metal pollution and with SfG values of <5 J $g^{-1}h^{-1}$ in Restronguet and Percuil, they appear to have insufficient capacity to grow, reproduce and maintain a viable population (reflected in the BCI for mussels from Restronguet but not in that for those of Percuil). Pill and St. Just have moderate stress values, and Mylor is apparently unstressed. The Bermuda study gave values of 3-10 J $g^{-1}h^{-1}$ for SfG; in the Oslo workshop and its study of industrial wastes, clean reference mussels had a SfG of approximately 10-12 J $g^{-1}h^{-1}$ and hydrocarbon and TBT polluted sites were 3-4 J $g^{-1}h^{-1}$; but in comparison the clean reference site in the Shetland study was about 24 J $g^{-1}h^{-1}$ and polluted sites 8-20 J $g^{-1}h^{-1}$. Widdows & Page (1993) found control values of 13 J $g^{-1}h^{-1}$ and TBT/DBT stress of -8 to 10 J $g^{-1}h^{-1}$. In the

Bermuda contamination gradient, using the mussel *Arca zebra,* reference values were 8 and 9 J $g^{-1}h^{-1}$ and pollution values of 3 to 8 J $g^{-1}h^{-1}$; these were for tissue concentrations of 68-86 mg g^{-1} zinc and 3-7mg g^{-1} lead and 5-9 mg g^{-1} copper.

Respiration rates.

It is difficult to interpret the significance of an alteration in the rate of oxygen consumption. For instance, there is conflicting evidence concerning the effects of petroleum hydrocarbons on the respiration rates of aquatic organisms. The direct effect of hydrocarbons is to enhance oxygen uptake and the observed decrease in respiration is largely the result of suppression of activity and partial closure when the mussel is exposed to higher concentrations (Bayne *et al.* 1985). Hence it is important not to consider individual responses such as respiration in isolation from other biological functions which act as covariants. Widdows & Page (1993) found enhanced respiration with increased TBT concentration with a decline only at higher concentrations which results from a cessation of pumping. The enhanced respiration rate with TBT reflects the primary mechanisms of toxicity of TBT which is to bind and inactivate membrane bound enzymes, then at higher levels, TBT exerts a neurotoxic effect on the lateral and latero-frontal cilia on the gills which are responsible for pumping and filter-feeding respectively which are under neural control (Stefano 1990). However, TBT is unlikely to be a factor in the Fal Estuary as Mylor Creek has the greatest boat activity and had the highest SfG value.

Knowledge of the effect of pollutants on respiration varies. In mussels different metals may affect oxygen consumption differently, *e.g.* an increase with silver (Thurberg *et al.* 1974) and a decrease with copper (Brown & Newell 1972, Scott & Major 1972). The same metal may also increase or decrease respiration depending on concentration or the animal species

(Muller 1979 in Bakke 1988). The decrease in oxygen consumption in Fal Estuary mussels suggests that the suite of metals present depressed oxygen uptake, either directly or through lower activity. Subtle differences in activity, enough to change respiration, are difficult to detect in *Littorina littorea* (Bakke 1988) which makes it uncertain as to whether differences in respiration are due to differences in behavioural response or effects on metabolism. As Bakke (1988) suggests suppressed respiration would tend to counteract a negative effect of pollution on feeding and assimilation in the SfG equation, making SfG less sensitive as an index of pollution than its component processes .

Possible errors.

It is difficult to explain the observed SfG values with respect to the heavy metal gradient. However, typical of this technique, specimens were selectively chosen with shut or slightly gaping mussels rejected and this may bias the data. Flow rates in laboratory experiments could have been a source of error; flow rates through each chamber were maintained constant at approximately 180 ml min^{-1} estimated to be low enough to record a significant difference between the inflow and outflow particle concentrations, yet sufficient to prevent recirculation of the water by the mussel (Widdows & Johnson 1988). The appropriate flow rates depend on the size and geometry of the experimental chamber and the clearance rate of the mussel, which can vary with body size and with physiological and environmental conditions. As a general guide it was suggested (Widdows & Johnson 1988) that flow rates through each chamber of the system should be approximately two to three times the clearance rate of the mussels, *i.e.* the particle concentration in the outflow should not be less than 50% of the inflow concentration.

Heavy metals.

59 mg kg^{-1} mussel tissue is the critical level of accumulated copper ultimately lethal to *M. edulis*, regardless of concentration of copper in the water or exposure time (Martin 1979). In contrast to petroleum hydrocarbons with no threshold effect, copper exerts sub lethal effects on feeding and growth rates of *M. edulis* over a relatively narrow range of water concentrations (less than one order of magnitude) with a threshold effect at around 5 to 10 mg copper l^{-1} and a marked inhibition at 20 mg copper l^{-1} (Manley *et al.* 1984; Redpath 1985, Widdows & Johnson 1988). If threshold values are not reached in the Fal this could imply that the effect observed in all creeks except Mylor, were due to another unidentified contaminant, possibly hydrocarbons from Falmouth. Previous studies showed that mussels exposed to diesel oil at concentrations of 30 mg l^{-1} reduced their clearance rates by about 30% (Widdows 1985). However, such an effect would have been expected at Mylor Creek as it is closest to Falmouth, but mussels from there were very unstressed with a high growth potential. 20 mg l^{-1} copper causes the cessation of shell growth in small (15 mm) mussels (Redpath 1985) and reduction of clearance rate by 50% (Manley 1983). In this study Fal Estuary copper concentrations were higher by orders of magnitude. The Oslo workshop reported that metal concentrations in tissue of *M. edulis* showed little variation (14.3 - 17.0 mg kg^{-1} copper per dry weight tissue) along Langesundfjord and were not sufficiently higher to cause observed effects on SfG (Widdows & Johnson 1988). Values in the Oslo study were slightly higher than in mussels from the Tamar estuary (Widdows *et al.* 1984).

Natural environmental variables.

The observed effects on clearance rate and SfG of mussels cannot be explained by the natural environmental factors of temperature and salinity along the pollution gradient, and of course all physiological responses were measured under standardised conditions in the laboratory. However, being an estuarine environment suspended particles flowing over the mussel cages could have a long term influence on the health of mussels by a possible smothering effect. This investigation underlines the possible difficulties in the SfG technique and thus it may not be an appropriate one to apply in an estuarine environment.

Ecological relevance.

A decline in SfG represents a deleterious effect. Field and mesocosm studies have provided confirmation that the long-term consequences to growth and survival of individuals and the population can be predicted from measured effects on energy balance at the individual level (Widdows & Salkeld 1992b). In an 18 month mesocosm experiment, a littoral community was exposed to two oil concentrations. Mussels at the lower concentration had a slightly negative SfG (-0.35 J $g^{-1}h^{-1}$), resulting in a gradual population decline after 12 months, whereas mussels at the higher concentration had a severely negative SfG leading to a rapid population decline over 6 months. In the Fal, the observed decline is shown in Table 2.6 (Chapter 2): by May 1995 88% Restronguet mussels had died, 29% of Mylor, 10% Pill, 1% St Just and 1 % Percuil. By May 1996 there was 100% mussel mortality in Restronguet, 50% in Mylor and 15% in Pill. Mussel mortality in fact is highly correlated to the heavy metal gradient and body condition index values.

CONCLUSION.

There is no clear relationship between SfG and heavy metal levels in the selected creeks of the Fal estuary. This approach has not demonstrated a sensitivity to the persistent high environmental levels of heavy metals in the Fal estuary, with the biological system showing no accurate reflection of the persistent gradient present. Feeding responses were related to final SfG values, which would be expected to have consequences at the population level. However, the physiological component of respiration rate correlated with the heavy metal gradient, as did body tissue weight. The method produced a general response to the total pollution and natural environmental stimulus, but without specificity in terms of linking ultimate values of growth potential to measured environmental factors and showed no discrete discrimination of sites.

CHAPTER 6

Larval Bioassay: Growth And Survival Of *Mytilus edulis* Larvae

INTRODUCTION

Bioassays

Bioassays, or toxicity tests, are experimental procedures in which organisms are exposed to different substances or combinations of substances to determine concentrations that affect them adversely. The period of exposure may be short (acute tests not involving a substantial portion of their lifespan) or longer term (chronic tests covering a substantial portion of the lifespan) (GESAMP 1995). Measured effects are either lethal (mortality) or sub-lethal (for instance impaired growth, irregular development, abnormal embryonic development, reduced reproductive output, and decreased moulting success) biological responses. Commonly-used tests are those that measure mortalities in marine fish, copepods, echinoid gametes, crustacean larvae and bivalve molluscs. Recently, cryo-preserved oyster and clam larvae have been used (McFadzen 1992) and are helpful for standardisation of such tests.

Assays are normally a detection of water quality but they can be used on sediment samples, for which the most common tests involve measuring survival of oyster larvae to extracted pore water (Thain 1992, Butler *et al.* 1992). However, where only subtle contaminant

effects are of concern sediment grain size may be a potential interference. Bioavailability of contaminants varies markedly and is difficult to predict in sediment (Knezovich & Harrison 1987). Standardisation of pore-water extraction is still experimental (GESAMP 1995). Recent applications of toxicity testing have been to sea surface microlayer (Hardy & Cleary 1992, Karbe 1992) where significant toxicity was detected. However, to what extent organisms in nature are actually exposed to this layer and hence the relevance of this approach, is debatable.

Routine bioassays of water quality provide a realistic means for detecting the effects of new toxicants in seawater, and for monitoring the integrated biological effects of contaminants and variables that influence their toxicity in seawater (Stebbing 1985). The origins of this approach lie in the accidental discovery that the developmental success of larvae of polychaetes and echinoderms used for settlement behaviour and metamorphosis experiments changed when the seawater came from different water masses, identified by characteristic planktonic indicator organisms (Wilson 1951). The larvae were used to provide an index of water quality, and the technique was applied to the identification of factors responsible for the differences in water quality (Wilson & Armstrong 1961). Woelke (1967) developed an oyster larval bioassay in which the index of water quality is the percentage of larvae that develop abnormally 48 hours after fertilisation. He applied this to waters polluted by pulp mill effluents (Woelke 1968). Connor (1972) showed that a range of invertebrate larvae were generally sensitive to toxicants at concentrations about two orders of magnitude lower than their adults. Many larval stages have the advantage that they are 'lecithotrophic' thus avoiding the problem of providing food and thereby avoiding experimental stress due to lack of food (Stebbing 1985).

Rearing mussel larvae

Any attempt to rear mussel larvae must include the acceleration of gametogenesis in the adult, inducing the adults to release ripe gametes (spawning), the maintenance of the larvae and their protection from disease. In attempts to secure a reliable supply of adults in spawning condition, techniques have been developed to accelerate gametogenesis by gradually raising the ambient temperature (Loosanoff & Davis 1950). Bayne (1965) obtained *Mytilus edulis* in a condition in which they were responsive to a spawning stimuls by raising the water temperature from 7 to 13 ^{o}C over 25-30 days. The required period for the conditioning of the adult stock in this way is of course dependent on temperature and on the stage of gametogenesis reached by the population. Other methods for inducing *M. edulis* to spawn include the administration of a mild electric shock, injection into the mantle of 0.5 M potassium chloride or ammonium chloride, mechanical irritation of the posterior adductor muscle, and exposure either to a rapid change of temperature or to the genital products of other individuals (Bayne 1976). There is a commonly observed latent period between stimulus and spawning suggesting an indirect action on the spawning process. The stimuli act through the follicle cells of the ovary initiating the secretion of a substance that causes the oocytes to begin the meiotic division and the stem that connects the occyte to the follicle wall to breakdown. Spawning would then follow automatically. Electrical stimulation and treatment with cations may also cause 'cytoplasmic maturation' of the sperm.

In spawning, eggs and sperm are discharged directly from the genital ducts to the exterior, and fertilisation occurs in the water. Ciliary activity in the ducts appears to be the main propulsive force for ejecting the gametes. Fertilisation of the eggs of *M. edulis* can be

delayed for 6 to 11 hours after release over the temperature range of 8 to 16 °C. The last sperms loose their motility many hours later. Successful fertilisation occurs over a wide range of sperm concentrations, with the optimum being 1000 sperms per egg. An egg concentration of more than about 20 eggs per ml has an adverse effect on the success of development (Sprung & Bayne 1984). In *M. edulis,* fertilisation occurs successfully over a temperature range from 5 - 22°C and in all salinities from 15 - 40 ‰. Environmental requirements for embryonic development may be limiting especially to estuarine populations of mussels. Unlike fertilisation, cleavage and early embryogenesis have a limited tolerance to environmental change. Bayne (1965) found that the development of the trocophore of *M. edulis* occurred successfully only within a salinity range of 30 - 40 ‰ and temperatures of 8-18°C. During the veliconcha larval stage there is considerable growth in size from 100 to 250 µm in shell length. Cell differentiation is limited to the velum (swimming organ), including its retractor muscles and the apical plate, to the alimentary system, the nerve ganglia and the cells of the mantle, which secrete the second shell. The larva then acquires a pair of pigmented eye-spots and soon afterwards develops a foot. This pediveliger stage immediately precedes settlement and metamorphosis into the adult stage.

Spawning and life-history

Mussels are plankotrophic and their reproductive 'strategy' is one of high fecundity, small eggs, external fertilisation and a pelagic larva that feeds on the phytoplankton. The sexes are separate (gonochoristic), unlike scallops and oysters. The gonad tissue, testis or ovary, is formed within the mantle, causing it to assume a characteristic colour as spawning approaches (the testis is whitish and the ovary orange). Fertilisation is external with the sperm and eggs released from minute pores on the inner surface of the mantle into the mantle cavity and out through the exhalent opening. Sexual maturity occurs after one year

of age. Spawning is seasonal, broadly in summer often with March-May and July-August peaks, usually with less than 50% of individuals in a population spawning at any one time.

Early life-history - Fertilised eggs, 70 µm in diameter, become swimming trocophore larvae within 12 hours. After about 2 days the trocophore has become a veliger larva (D-larva) with a D-shaped shell < 120 µm long, the prodissoconch I shell secreted by the shell gland. The ciliated velum is responsible for feeding and locomotion. The next stages are the veliconcha, after the secretion of the prodissoconch II shell secreted cyclically by the mantle, and the pediveliger, after the development of the foot. After a few days larvae feed on particles < 9 µm in diameter. The velum collects food and cilia move it to the mouth. Algae are the main food but bacteria can be used although they lack the long-chain polyunsaturated fatty acids essential for bivalve growth. After 15 to 35 days, depending on variables such as available food, temperature, and salinity, the larva metamorphoses with secretion of the adult shell, the dissoconch, into a young post larva called a plantigrade. The maximum growth rate is 12 µm per day and this occurs in the 16 - 22 °C temperature range and at 30 - 35 ‰ salinity; this gives a maximum larval life of 21 days. At 6 °C it will take 56 days. Growth rates begin to decline at salinities of 20 ‰ and ceases below 15 ‰. The pediveliger stage is ready for settlement when it reaches 300 µm.

To settle, the pediveliger becomes positively geotactic and negatively phototactic. Chemical and other stimuli are used to select areas where other mussels are established and where there is adequate water circulation. After bysuss attachment metamorphosis occurs and the larva becomes a juvenile (plantigrade).

The larval survival bioassay

Wilson (1951) and Wilson and Armstrong (1961) used larval development of invertebrate species (echinoderms and polychaetes) to identify different water masses. The bioassay, as a monitoring tool, was initially developed by Woelke (1972) for use with oysters. Subsequently, researchers used both embryonic and larval stages of various species to assess water quality (Woelke 1967, 1972, Kobayashi 1971, Connor 1972, Thain 1991). Stebbing and Pomeroy (1978) found that laboratory cultures of cloned hydroids overcame reproductive seasonality of test oysters etc. Cryo-preservation of larvae have been used similarly to eliminate reproductive seasonality, as well as having the advantage of minimising genetic heterogeneity between tests and maintaining viable larvae for use over unlimited time periods. McFadzen (1992) applied this technique with oysters to investigate the pollution gradient in the German Bight during the Bremerhaven workshop. Hence, water quality bioassays using the pelagic larval stages of several taxa of marine invertebrates have been routinely used to monitor the integrated biological effects of contaminants and the variables that influence their toxicity in sea water (Stebbing 1985).

'Deterioration in water quality' implies that a change in chemical, physical, and/or biological composition has occurred which is potentially harmful to aquatic organisms. A bioassay to measure such deterioration should be based on a response by an organism which clearly represents a harmful effect at both the individual and population level of organization. The organism response used in this bioassay is the ability of the mussel embryo to develop normally and reach the 'D' - shaped larval stage (at which the paired hinged shells can be seen) within 72 hours. Although the exposure time is short, it encompasses a period of intense cellular activity during which the impairment of a number

of critical physiological and biochemical processes may result in poor growth and development. The response measured is, therefore, similar to that used in other early life stage tests which record growth and development, and it has the advantage that exogenous feeding is not required, thus eliminating this source of variation in the tests results (Thain 1991).

Aims

The aim of this technique was to investigate the growth and survival of larvae of *Mytilus edulis* when exposed to heavy metal contaminated water of the Fal estuary. The possibility of using mussels from the field site was assessed as well as the application of the technique to larvae reared from a clean site location and exposed in the laboratory to contaminated water from the Fal Estuary.

MATERIALS AND METHODS

Sampling.

Larvae were reared from adults of *M. edulis* collected from a known 'clean' site at Whitsand Bay, Cornwall. They were also reared from mussels collected originally from Bull Hill in the Exe estuary, then transplanted to sites in the Fal estuary and left in location for one year. The precise sites in the chosen study creeks have been described in Chapter 2. Mussels sampled were 40-50 mm in length and returned in a cool box to the lab where they were cleaned of all epibiotic growth. Whitsand Bay mussels were used as controls.

Larval culture.

Experiments were conducted during those months estimated to be normal spawning periods for natural *M. edulis* populations, that is April and May (as well as the months previous and subsequent to these so as not to miss their reproduction cycle). On return to the laboratory, individual mussels were placed in one litre glass beakers in a controlled temperature room at 20°C. Any attempt to rear mussel larvae must include the acceleration of gametogenesis in the adult, inducing the adults to release ripe gametes (spawning), and the maintenance of the larvae. To induce spawning the protocol of Pascoe (pers. comm.) was followed. Mussels were just covered with filtered sea water at 30°C which immediately fell to 28°C then gradually to 20°C over one to two hours. Spawning usually occurred after 15 to 60 minutes, or not at all. Clean natural seawater was used rather than artificial seawater as the latter varies in quality and often produces inconsistent results in control embryo development (Thain 1991).

For fertilisation, 1 ml of sperm was added to 100 ml eggs and kept at 15°C awaiting addition of test water samples some 12 hours later. After mixing they were left without aeration for 1-2 hours during which time a sample was examined under a microscope to confirm that the early stages of cleavage were occurring. Success of fertilisation was assessed by checking the existence of adequate numbers of viable veligers under a microscope. Once spawning occurs, fertilisation and the culture of early embryonic stages follow, but if larvae become dependent on planktonic food, cultivation is more problemmatical. Hence, the bioassay was conducted on larvae at an early stage while they were self-sustaining.

Bioassay.

The assay was successfully performed on three dates: 4th April, 21st April and 2nd May 1995. To start exposure, eggs at an early stage of cleavage were added to each test sample (100 ml). For the control, filtered sea water was added to the glass beakers containing healthy veligers. Samples were equilibrated at 20°C for larval incubation. All samples were maintained under static conditions and stored at 20°C for 60 hours. No aeration is necessary unless the samples have a high oxidizable organic content (Thain 1991). Other studies (Woelke 1972 and McFadzen 1992) used a 48 hour timescale, but in the present study, the larvae had not developed enough by this time and so incubation was extended to 60 hours. At the end of exposure each sample was treated with 4% buffered formalin to preserve the larvae for analysis.

Microscopic examination of larvae was conducted on amounts of each sample extracted at random and deposited on Sedgewick Rafter counting cells. Survival was assessed as number of viable D-shape larvae observed in the first 400 larvae encountered. Other workers have counted the first 50 (McFadzen 1992) or 100 (Thain 1991). In the first experiment additional degrees of abnormality of larvae were recorded: Normal D-shape, slightly abnormal, abnormal, severely abnormal and undeveloped fertilised egg. Such abnormalities consisted of either nicks in the edges of shells, reduced or nil development of shell valves or distorted clumps of cells which developed no further towards a D-shaped veliger.

Calculation of results - the number of 'abnormal' embryos was calculated as 400 minus the number of normal D-shaped larvae. 'Abnormal' included those eggs not fertilized, and

those which died at an early stage of development or became malformed. Statistical analysis was by two-way ANOVA.

RESULTS

Larval viability.

Inducing spawning was problemmatical. Spawning of mussels from the creeks in the Fal estuary was unsuccessful on all occassions, except for one male from Mylor, which died one hour later, and one female from Percuil, which produced abnormally shaped, void ova, some with stalks still attached. Dissection of Fal mussels revealed that the mantles were usually extremely thin and devoid of gametes. Hence a water quality bioassay based on survival of larvae derived from these mussels was not possible. Instead, induced spawning of mussels from the clean site at Whitsand Bay was relied upon and successful on several occassions, and these gametes, were immediately transferred to clean seawater at 15°C and checked for their viability *i.e.* motility of sperm and roundness of eggs. Sperm are identified by their milky appearance and eggs by their granular appearance in the water.

Survival.

Bioassay results are expressed as the mean number of normal D-shaped larvae to develop in each of the test samples and in the control. All data are presented in Table 6.1 and Figure 6.1. Figure 6.2 shows a healthy D-larva and an undeveloped embryo.

Site	Exp. 1	Exp. 2	Exp. 3	Mean
R	1.0	0	0	**0.34 ± 0.58**
M	29	36	44	**46 ± 7.5**
P	47	46	22	**39 ± 14.1**
J	26	54	13	**31 ± 20.9**
E	41	41	52	**45 ± 6.3**
Control	62	74	78	**71 ± 8.3**

Table 6.1 Percentage survival of larvae on different experimental dates.

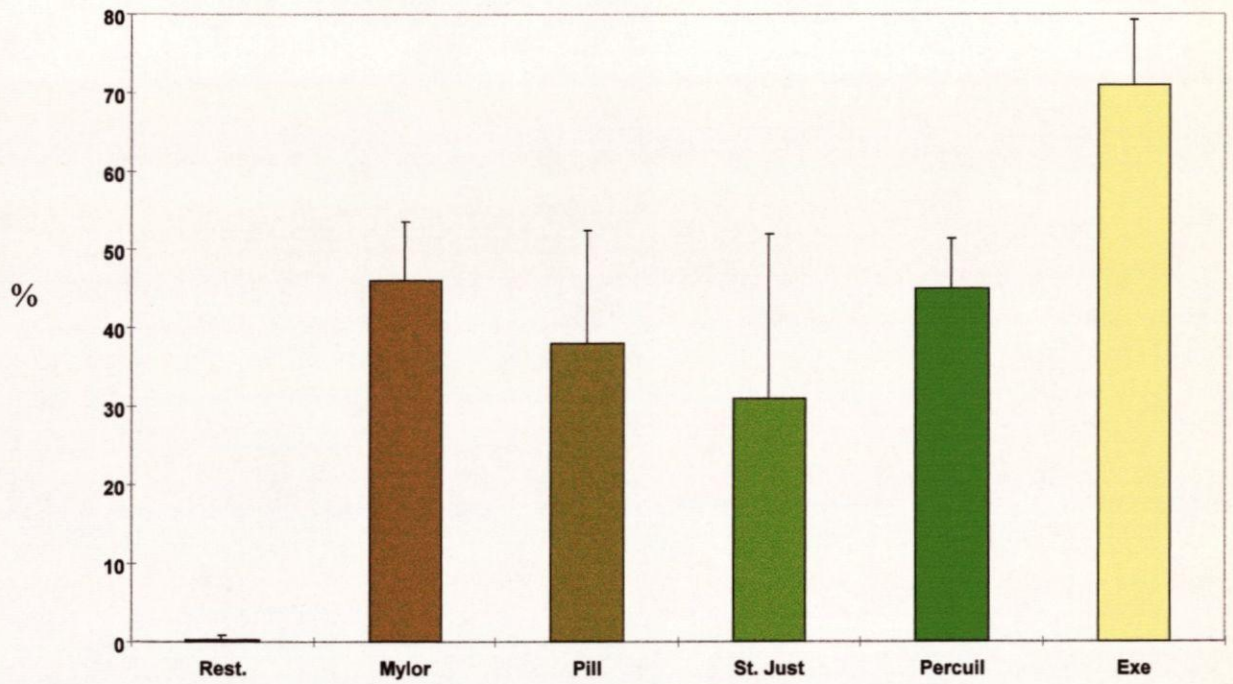
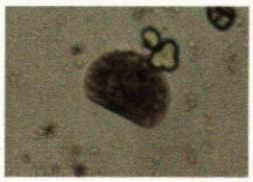


Figure 6.1 *Mytilus edulis* larval survival: (Bars indicate standard deviation).

(a)



70 µm

(b)

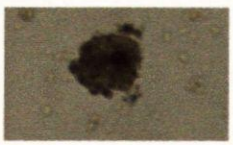


Figure 6.2 *Mytilus edulis*
(a) Healthy D-shape larva (b) Undeveloped / abnormal larva.

Experiment 1 - The mean survival of control larvae was relatively low at 62% in contrast to 96% viability of oyster larvae used by McFadzen (1992). The survival of larvae exposed to water collected from Restronguet creek was extremely low at 1.0% and significantly different ($p < 1\%$) to all other creeks. The other four creeks showed significantly different ($p < 5\%$) survival rates to Restronguet and varied from 26 - 47%. These results for the four creeks did not reflect the heavy metal gradient present in both the sediment and mussel tissue. The four showed no significant differences in survival between sites, with the second most polluted creek, Mylor, being very similar to the fourth most polluted, St Just, with values of 29% and 26% respectively. The third most polluted creek, Pill with a 47% survival rate was very similar to the least polluted creek at 41%.

The breakdown of data into degrees of abnormality (Table 6.2) show that more than 50% larvae exposed to a water sample from Restronguet Creek did not develop past the stage of a few-celled embryo of distorted form illustrated in Plate 6.1. In comparison, samples from the other sites as well as the control showed only 20 - 25% distorted. The control larvae

had the lowest proportion of abnormal forms, and the other four creeks varied as they did with healthy D-shaped veliger larvae.

Site	Healthy D	Slightly abnormal	Moderately abnormal	Severely abnormal	Embryo only
R	1	11	32	0	56
M	29	37	10	3	22
P	47	12	23	1	18
J	26	32	16	1	26
E	41	23	12	2	27
Control	62	12	1	1	24

Table 6.2 Percentage breakdown of different abnormalities of larvae from experiment 1.

Experiment 2 - The survival of control larvae was more successful at 74% than for the previous experiment. No larvae survived in the Restronguet water sample. Through Mylor, Pill and St Just samples survival was 36%, 47% and 54% respectively following the heavy metal gradient. However the sample from Percuil, which had the lowest contamination, had an intermediate survival rate of 41%.

Experiment 3 - 78% control larvae reached the normal D-shaped form. Once more no D-shaped veligers developed in the Restronguet sample. There was poor development of healthy larvae in the St. Just sample at only 13%; Pill had 22%, Mylor 44% and Percuil 52% successful survival rates. Hence, again data did not correspond to the heavy metal gradient.

Experiments averaged - Restronguet samples were significantly different ($p < 1\%$) to those from all other sites with, at 0.34%, lower successful larval development. The remaining four creeks varied between 31 % and 45 %, were not significantly different to one another and did not accurately reflect the heavy metal gradient (Chapter 8). Control larvae development was significantly more successful at 71 %. Two-way ANOVA shows significant differences between sites ($p = 0.001$) and a difference between dates ($p = 0.0007$).

DISCUSSION

Summary

The aim of the current investigation was to assess the biological effects of water contaminated with heavy metals, derived from a known contaminated sites in the Fal Estuary, on *M. edulis* larvae. There was no success in rearing larvae from mussels from the Fal estuary, so clean site mussels were used to produce larvae against which test water samples could be assayed. The results from the survival data do not correspond to the known heavy metal gradient. However, larvae responded to the extreme loading of metals in Restronguet Creek, exhibiting < 1% survival of healthy D-shaped veliger larvae. They were also affected to a degree in water from the four less polluted creeks.

Spawning difficulties

The embryonic stages of bivalve larvae, usually those of oyster, have been routinely used to assess water quality (for instance Thain 1991, McFadzen 1992). This early developmental stage has been used because it is convenient rather than because it has any preferred sensitivity (McFadzen 1992). The major disadvantage is that there is limited seasonal

availability of this particular life stage, a difficulty also met in this study in inducing mussels to spawn. Mileikovsky (1970) discussed some of the factors that affect the timing of the occurrence of larvae in near-shore areas. He identified differences in the spawning time of the adults due to the zoogeographical origin of the species, the correlation of these spawning times with local seasonal temperature changes, and the operation of various types of lunar and diurnal spawning rhythms. In some cases it is possible to initiate larval release only twice in a lunar month at the time of spring tides (Kobayashi 1971). Some workers have used potassium chloride injected into the posterior adductor muscle of *M. edulis* (Bayne 1972) as a spawning stimulus. However, the animals must still be ripe and timing can vary from year to year and from location to location. Cryo-preservation techniques have been developed to overcome such problems, which also result in reduced genetic heterogeneity between samples allowing bioassays on siblings of identical age over several seasons (McFadzen 1992), but makes the assay more expensive. Freshly reared larvae were used in this study.

Larval size

A common observation during laboratory culture was the large size range of sibling larvae reared under identical conditions. This has been related to adult condition. Physiological differences between eggs from the same female could arise from unequal distribution of nutrients to different parts of the gonad. Variation between eggs from different parents could be due to differences in the physiological condition of the adults. Bayne (1972) held adult *M. edulis* under conditions of considerable physiological stress. The adults were induced to spawn and the development and growth of the embryos and larvae compared, under standard conditions, with larvae from non-stressed adults. Embryos from stressed adults showed increased abnormalities during development. The chances of larval survival

are determined by egg viability which is reduced under stress. Bayne *et al.* (1978) recorded a reduction in the organic matter per egg of *M. edulis* under stress in the laboratory, and Helm *et al.* (1973) found reduced lipid content in *Ostrea edulis* both resulting in a slower growth rate of larvae.

Size of larvae can affect uptake of contaminants. Manahan and Crisp (1982) studied the uptake of dissolved organic material by veliger larvae and postulated that the velum was the site of uptake rather than the digestive tract because of its larger available surface area. Manila clam larvae (*Tapes philippinarum*) were more sensitive than larvae of the Pacific oyster (*Crassostrea gigas*) which was associated with their size and surface area (McFadzen 1992). *M. edulis* larvae are the approximate size of *Crassostrea* larvae at around 80 µm, wheras the larvae of *T. philippinarum* are approximately 130 µm.

Use of other organisms

Hydroids have been used to avoid some of the problems of working with larvae. Their culture is straightforward and they can be maintained for indefinite periods with *Artemia* nauplii. Hydroids are sessile which simplifies their handling in experiments, and as they reproduce asexually, material for a complete experiment can easily and quickly be grown from a single hydranth (Stebbing 1985). Those who manage environmental levels of contaminants however are not as concerned with the survival of hydroids as with ecologically or economically more important species. The possibility of translation of sensitivities to toxicants was established quite early (Shaw 1961) via a systematic study of the effects of different toxicants on a range of organisms. This showed that the relative toxiticities of metals remained constant for each organism.

Control abnormality

This investigation involved testing larvae which even in control samples had only 60 - 80% viable veligers present, as compared to 96% viable content of the cryo-preserved larvae (McFadzen 1992). Other studies have shown developmental abnormalities from fertilised eggs to the D-shell larvae in controls to be as great as 50% (Thain 1991, Utting and Spencer 1991). Such high control abnormalities may be hereditary (Bayne 1972) with no means of detection prior to the onset of the bioassay. However, once more the technique of cryopreservation of D-shell veligers from successful spawnings of a high developmental success can be used to provide material which is indefinitely available for use in bioassays.

Sources of error

One source of error may be the particle loading of the test samples. It may have been necessary to remove particulates and suspended solids which can affect embryo development (Davis & Hidu 1969). The exact particle size and particle loading which affect embryo development are not known, but they are unlikely to be under 10 µm in size and in excess of 300 ppm loading (Thain 1991). In estuaries where turbidity is typically high, it is a serious factor for consideration. Thain (1991) does not recommend filtration as it may remove some of the dissolved contaminants. For small numbers of samples centrifugation is recommended.

CONCLUSION

In conclusion, this investigation has illustrated that *M. edulis* larvae are sensitive to the extreme heavy metal contaminated water from the Fal estuary. The assay detected the extreme site of pollution, Restronguet Creek, as being significantly different from other less polluted sites, but it did not accurately detect the underlying heavy metal contamination gradient. It was a simple and effective technique once larval spawning and rearing was successful. Plankotrophic larvae such as *Mytilus* suffer considerable mortality and loss during dispersion. Any reduction in the larval quality or extension of the larval period, due to reduced rates of growth, represents an added drain on recruitment potential and hence on ecological fitness.

CHAPTER 7

Lysosomal Membrane Damage In *Mytilus edulis*: The Neutral Red Retention Assay.

INTRODUCTION

There has been an increasing requirement for early detection of chronic change in marine environmental impact assessment, and investigations at the subcellular level can reveal alterations at an early stage of response before integrated cellular damage shifts to the level of organ or whole animal physiological processes (Moore & Simpson 1992). Recent developments have seen the use of lysosomes which are sub-cellular organelles, mainly involved in the intracellular digestion of food, cellular defence mechanisms, protein and organelle turnover and regulation of secretory processes. They exhibit changes in their structure and function (Moore 1985), and many advocate using them in studying environmental pollution impact (Cajaraville *et al.* 1995, Lowe *et al.* 1995a and b).

Bivalve blood cells (haemocytes) are particularly rich in lysosomes (Moore 1985). Since these lysosomes also accumulate several xenobiotics it is possible to measure their characteristics such as volume, surface, size and numbers in isolated cells and to use changes in these parameters as biomarkers of environmental pollution. An endpoint is

lysosomal membrane condition *in vitro* as indicated by neutral red uptake (Borenfreund & Puerner 1985, Lowe *et al.* 1992) and this approach is the subject of this Chapter.

This technique was examined recently at the Bremerhaven workshop and agreement was found between techniques involving lysosome membrane stablity and other levels of biological organisation. Thus there is support for this approach especially as some suggest (Moore 1990b) that it has potential to link pathological changes at the molecular and sub-cellular levels of organisation to impairment of the physiological performance of the whole animal.

Cellular assays

Methods described to evaluate the effects of contaminants on intact cells for biological impact studies mainly on marine fish species involve techniques of gross pathology and histopathology (Lowe *et al.* 1992) developed from pharmacological research into effects of drugs on cells and tissues. The effects of chemicals causing disease and death in organisms can be seen initially at the cellular level, and so individual or cultured cells can be used for bioassays *in vitro* as a simple indicator of the health of the organism (Grisham & Smith 1984). These tests, in which whole organisms are not sacrificed have the advantage that follow-on assays can be conducted on the same cells or animals, thereby eliminating consequences of genetic heterogeneity between samples.

Pharmacological studies have used dye extraction from cells and estimated the proportions spectrophotometrically. Other assays use acradine orange, a cationic probe, detected fluorescently using blue light excitation (Lowe *et al.* 1992). Thus cell biology can play an

important role in understanding the effects of xenobiotics on organisms because the cell is the site of xenobiotic accumulation, metabolism and reaction. It can provide a useful quantitative tool which, with the aid of computer assisted image analysis, densitrometry and flow cytometry, can quantify changes in cellular and tissue structure and functions.

Cells provide a link between molecular and biochemical events and whole animal events within living organisms (Cajaraville *et al.* 1995), and hence can be used in biomarker studies. The main advantage of using cells instead of animals in toxicity testing and environmental monitoring is the great reduction in the number of animals needed for the analyses. Other advantages of using these methods for monitoring programmes include high reproducibilty, rapidity and cost effectiveness as well as the ability to sample individuals more than once without destroying the animals.

Discovery of lysosomes

Pitt (1975) showed that cells could be dismantled under controlled conditions and separated into the various components by differential centrifugation. The cell homogenates contained many more smaller particles than were already known (nuclei and mitochondria) including ribosomes and fragments of the endoplasmic reticulum called 'microsomes'. Mitochondria and 'light' mitochondria had been separated from rat liver preparations. The latter group contained acid hydrolases and were subsequently called lysosomes, *i.e.* lytic bodies. These cell particles were 0.2 - 0.4 μm in diameter. Pitt (1975) also reported that these enzymes showed 'latency' since they failed to hydrolyse substances until they were released from the particles by osmotic shock, detergents, or by freezing and thawing, treatments known to disrupt cell membranes (see Figure 7.1).

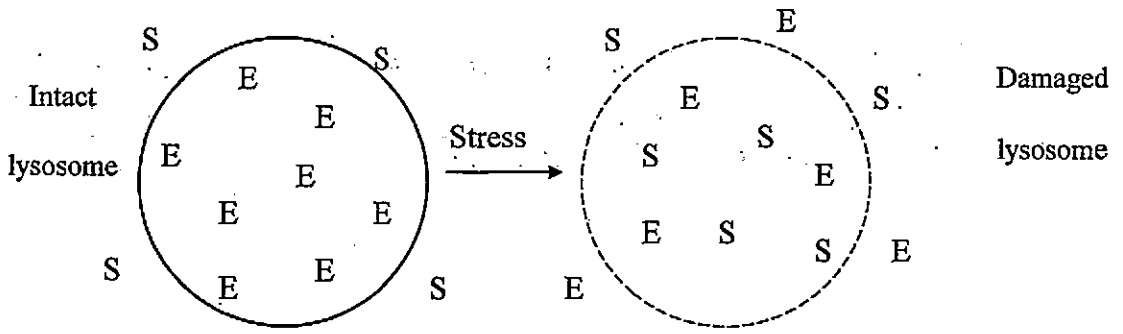


Figure 7.1 Early model of the lysosome based on the original ideas of de Duve (1969) (source Pitt 1975). The membrane in the normal state is impermeable to outward passage of the enclosed enzymes, E, and to inward flow of substrates, S, which must gain access to the interior of the organelle before hydrolysis will occur. Membrane permeability may be altered by various means when the enzymes become activated. During cell injury and disease enzymes may be liberated and cause varying degrees of metabolic disturbance.

After disruption of cell membranes and centrifugation, previously particle-bound latent enzymes were active as particle suspensions in the supernatant fluid. These included an organelle that possessed great potential for metabolic disorganisation with enzymes capable of degrading almost all the important macromolecules of the cell, separated from cytoplasmic substrates by a delicate single lipoprotein membrane (De Duve 1969). Not until later was the importance of lysosomes in cell injury, disease and death realised. Kidney lysosomes from rats, were able to accumulate foreign proteins and act in detoxification leading to a connection being made between lysosomal digestion and endocytosis. This important period moulded current ideas about the nature of sub-cellular organisation which up till that time extended little beyond the concept of the cell as 'a bag of enzymes'.

Lysosomes

Lysosomes have diverse functional roles. Intracellular digestion is the main role of digestive lysosomes, ingesting and degrading foreign bodies by membrane diffusion (Dean 1977). Other functions include regulation of secretory processes, cell death, accumulation and sequestration of xenobiotics, regulation of amino acid transport into cells by degradative inactivation of carriers by lysosomal proteases, mediation of target tissue-specific hormone functions, protein and organelle turnover and cellular defense mechanisms (Moore 1982). They are also involved in reproduction, immune response and membrane repair. In bivalves, lysosmones play an important role in excretory systems where they are involved in metal accumulation and excretion. Moore (1982) in his comprehensive review noted that lysosomal protein catabolism is responsible for the generation of amino acids for intracellular osmoregulation in *Mytilus edulis*. It is this function which involves fluidity changes, or permeablity, in the lysosomal membranes. In their role as components of the immune response, blood cell lysosomes release acid hydrolases which degrade circulating pathogens. If the membrane is damaged it does not function properly, resulting in the unscheduled release of acid hydrolases which causes further damage to the cell. It is necessary to be aware of these 'normal' functions of lysosomes in the physiology of marine invertebrates to appreciate their additional role in stressed and polluted organisms.

Bivalve digestive and blood cells are some of the richest sources of lysosomes (Moore 1985; Cajaraville *et al.* 1995). In spite of morphological and biochemical heterogeneities, sufficient similarities exist between lysosomes from various tissues to justify their inclusion in a single group of particles rather than subdivide them into numerous separate groups of

organelles. This aids the present study as the majority of work done on digestive cell lysosomes is equally applicable to blood cell lysosomes.

Other techniques for examining lysosomal perturbation

There are various techniques for examining the stresses of the lysosomal compartment. For example, there are cytochemical procedures for determining lysosomal membrane fragiliy in terms of a labilisation period. This can be assessed by microscopical determination of reaction intensity for lysosomal enzymes in tissue sections (Moore & Viarengo 1987). Also, histological methods can be used where lysosome physiology is observed in tissue sections. However, such methods are time-consumming as extensive pretreatment of tissues is required. Furthermore, the methods require sacrifice of the target organism, therefore live tissue is not used. In contrast, methods such as the neutral red technique require little pretreatment, are non-destructive and changes in the vesicular events can be observed as live cells are used.

Development of the neutral red assay

Babich & Borenfreund (1987) demonstrated the use of neutral red dye during *in vitro* assays for contaminant induced damage in fish as only lysosomes in healthy cells take up and retain this supravital dye. The assay as conducted in this chapter was done for the first time on fish hepatocytes for the Bremerhaven workshop (Lowe *et al.* 1992) where it produced reliable results on a pollution gradient, and for the first time on **live** mussels from the Venice lagoon (Lowe *et al.* 1995a).

Until recently, evidence for damage to lysosomes by environmental contaminants in aquatic organisms had been obtained from tissue sections rather than living cells (Moore 1985, 1990b, Hinton 1989, Kohler 1989). Lowe *et al.* (1992) extended the study of xenobiotic induced lysosomal injury to living cells, using cells isolated from the livers of the flat fish, *Limanda limanda* (Dab). However, the disaggregation protocol for liver cells is a complicated process. Lowe & Pipe (1994) extended the work to pollution studies in mussels using the more readily accessible blood cells. They showed that lysosomes are the target for toxic action of hydrocarbon pollutants impairing their functional integrity and found supportive evidence that cell injury was apparent from increased accumulation of lipid and lysosomal hydrolase activity. Lowe & Pipe (1994) also found in clean site control mussels that neutral red was restricted to a few secondary lysosomes, whereas in contaminant exposed mussels, the probe was in numerous small lysosomes distributed throughout the cytoplasm.

Regoli (1992) investigated lysosomal responses to heavy metal pollution and to natural fluctuations, such as the effect of season on *Mytilus galloprovincialis* in Italy, and effects of transplantation on the digestive gland and hydrolase latency. Regoli found lysosomal membrane stability of transplanted clean mussels showed changes after only one week, to become comparable to native polluted mussels. For mussels transplanted from polluted sites to clean tanks of seawater he observed a slight, but significant, increase in membrane stabilty. He also found that mussels transplanted from clean to polluted sites developed enlarged secondary lysosomes.

Previous related studies

There are various field studies on the use of lysosomal responses as biomarkers of exposure to environmental xenobiotics in natural populations of marine molluscs. Besides Regoli's investigations with mussels (1992), Moore *et al.* (1987) found that *Littorina* near an oil terminal in the Shetland Isles displayed reduced stability of digestive lysosomes; Lowe *et al.* (1995a) used mussels to monitor the effects of a range of contaminants in the Venice Lagoon, Italy; Moore (1988) investigated *Littorina* and *Mytilus* in the North Sea for the Oslo workshop; Cajaraville *et al.* (1995) studied mussels and various contaminants in the Abra estuary in Spain; Krishnakuamar *et al.* (1994) looked at mussels from urban-associated sites in Puget Sound, Washington. There is less literature on blood cell membrane stability in natural populations of mussels and the neutral red assay.

The majority of work has been conducted on digestive cells, but the information is transferable; blood cells and digestive cells of the same animal do not have the same function, but the response of the lysosomes of each cell type does not differ with varying exposure. Lowe *et al.* (1995b) investigated the cellular responses to contaminant exposure, comparing both blood cells and digestive cells from *Mytilus* using the neutral red assay. They found no significant difference between lysosomal retention for the two cell types. This indicates that the contaminant effect is independent of the compliment of the acid hydrolases within the lysosomes and there may be a failure in a common controlling factor.

Lysosomal alterations have been reported in several marine invertebrates, such as mussels (*Mytilus edulis*) and periwinkles (*Littorina littorea*), exposed to xenobiotics such as polycyclic aromatic hydrocarbons (Lowe *et al.* 1981, Moore *et al.* 1982, 1985, 1986.) and

to heavy metals (Sternlieb & Goldfischer 1976, Harrison & Berger 1982, Viarengo *et al.* 1987, Regoli 1992).

The basis for the assay - neutral red & the proton pump

The neutral red retention assay is based on lysosomal damage possibly through impairment of the so called proton pump (Lowe pers. comm.). It measures the movement of the acidic pH lysosomal contents into the neutral cytosol using the dye as a marker of the efflux. Thus it measures a normal physiological process (Lowe *et al.* 1995b), one that may be damaged and affected by pollution and stress. The assay is based on the fact that lysosomes can become functionally impaired as a result of their abilty to concentrate environmental contaminants, such as heavy metals, and this increases the permeablilty of their membranes (Moore, 1992).

Alterations in the capacity of cells to take up the dye neutral red can be used as an indicator of cell damage, as healthy cells take up and retain the dye longer than damaged ones. This was done *in vivo* and measured spectrophotometrically (Borenfreund & Puerner 1985). Lowe *et al.* (1992) quantified the technique by observing lysosomes under a microscope from fish hepatocytes and mussel digestive cells (Lowe & Pipe 1994), then extended it to live mussel studies (Lowe *et al.* 1995a). The blood cells are very easy to obtain without harming the host and are robust, thus enabling the opportunity for further assays on the same animal.

Neutral red is lipophillic and so will freely permeate the cell membrane (Lullman-Rauch 1979). Within the lysosomes neutral red becomes trapped by protonization and can be observed by microscopy. The extent to which this molecular probe is retained is dependent on the pH of the lysosome as well as the efficiency of its membrane-associated proton pump (Seglen 1983), so any impairment of this system will result in a reduction of the dye retention. Any perturbation of the membrane structure and associated impairment of the ATP-dependent proton pump results in release of neutral red dye into the cytosol (Lowe *et al.* 1992). The contents of the lysosomes are acid and are maintained by the pump (Okhuma *et al.* 1982). A deleterious effect on the proton pump system will result in an increase in the pH of the lysosomal contents, and as the gradient across the membrane reduces so does the ability to retain the neutral red dye and the lysosomal contents plus the neutral red move more freely into the cytosol, as illustrated by Figure 7.2.

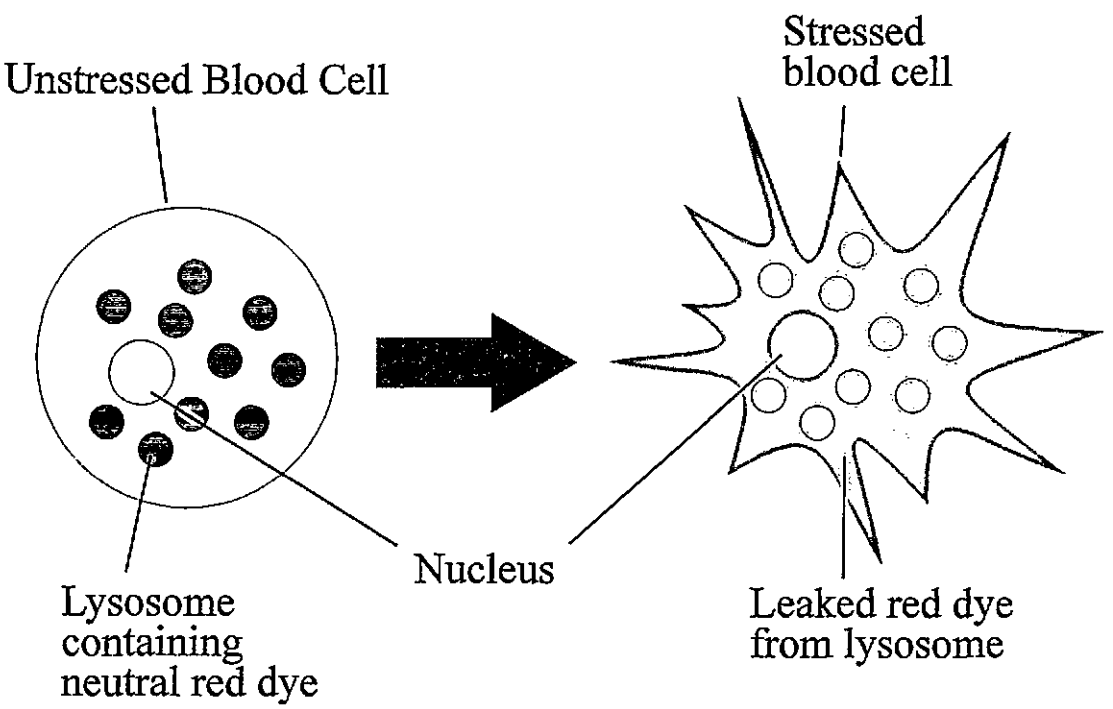


Figure 7.2 Diagrammatic representation of the use of neutral red dye to track lysosomal cell damage effects.

Effects of stress on lysosomes

Environmental stressors induce alterations in the ultimate effectiveness of lysosomal enzymes leading to disturbances in their normal physiological functions, and they also induce an increase in numbers of autolysosomes involved in digestive processes. These effects can have considerable consequences in marine invertebrates (Owen 1972). Lysosomal responses to cell injury due to contaminant exposure fall into three categories:

i. changes in lysosomal contents

ii. changes in fusion events

iii. changes in membrane permeabiltiy.

These can be exerted as observable changes in size and number of lysosomes. A reduction in size and number of lysosomes is probably related to excretion of lysosomes containing contaminants or their metabolic derivatives (Cajaraville *et al.* 1995). Lysosomal enlargement may be associated with increased lysosomal fusion processes. and can be an indicator of metal induced effects in molluscs. Howevér, size changes are variable. Larger, fewer lysosomes were reported by Marigomez *et al.* (1989), Lowe & Clarke (1989) and Moore & Clarke (1982) and were attributed to altered membrane stability leading to increased fusion of organelles. Etxeberria *et al.* (1994) found increasing zinc resulted in enlarged and more numerous digestive lysosomes in mussels, both in the field and in the laboratory. This can also be observed in field situations of low food availabilty, *e.g.* during winter.

Lysosomal protein catabolism is important in the generation of amino acids for intracellular osmoregulation in the mussel, and it is this function which involves permeability changes in the lysosomal membranes. The effect of contaminant-induced changes on the permeability of membranes results in interference with the normal processing of intracellular proteins and organelles (autophagy) and the intracellular digestion of pinocytosed food (heterophagy). The mechanism involves Golgi-associated endoplasmic reticulum giving rise to lysosomes (this was suggested as a biomarker at the Bremerhaven workshop (Kohler 1991)).

These assays do not identify the nature of the particular stressors or contaminants that are causing the cell injury. However, more specific information about the causative agents can be obtained by using tests for lysosomal accumulation of sulphydryl-rich metal-binding proteins, *e.g.* metallothioneins, which are induced by exposure to particular metals (Moore 1990a).

Role of metals in cells and their effects

Heavy metals such as iron, copper, zinc and manganese have essential roles in cellular metabolism by contributing 'prosthetic groups' activating enzymes, and are essential for the development or maintenance of lysosomal structural integrity. For instance, iron and copper are needed for the functioning of mitochondrial cytochrome-*c* oxidase. Optimal enzymatic activity of certain lysosomal enzymes is dependent of the presence of cations such as Fe_2+ or Co_2+ for the catalytic activity of *x* mannosidase (Sternlieb & Goldfischer 1976). But under different conditions or excessive concentrations the same metals may be detrimental, acting as inhibitors of enzymes or as cytoplasmic toxins. Normally, however, the uptake of cations from the blood, their distribution in the cell and excretion are regulated so that

homeostasis is maintained. Excessive amounts of metals in or around lysosomes may be caused by:

i. enhanced uptake

ii. diminished cellular excretion

iii. oversupply

and these can lead to alterations of lysosomal structure or enzymatic content (Pitt 1975).

Lysosomes act as protectors of cellular homeostasis through the sequestration and chelation of potentially toxic elements and as targets for uptake of potentially toxic elements. The stability of their membranes may depend on the concentration of certain metals such as zinc, lead and copper and may be enhanced or diminished by the effects of metal ions or salts with diminishment causing a release of hydrolases and apparent discontinuity of membranes (Sternlieb & Goldfischer 1976).

Mussels are relatively tolerant to many metallic and organic xenobiotics. This tolerance, however, does not mean that the animals are unresponsive; there is evidence for pathological reactions even to low concentrations of metal contaminants. The major response involves enhanced autophagy, a generalised response to stress. This is expressed as an increase in the volume of the lysosome contents, a swelling of the lysosomes and an increase in hydrolase activity. It is unclear whether the autophagic changes predispose the cells to deletion (Moore 1990b).

Metals associated with metal binding proteins (metallothioneins) may enter the lysosomal system for detoxification within normal protein turnover. Metals accumulate within lysosomes in a number of metal storing cell types such as bivalve digestive cells (Viarengo 1989). Thus, lysosomes are important organelles for metal sequestration and detoxification, but excessive concentrations may overload their storage capacity and cause changes in the integrity of the membranes (Sternlieb & Goldfischer 1976). Such reductions in membrane stability have been recorded after experimental exposure to copper and cadmium (Viarengo *et al.* 1987).

MATERIALS AND METHODS

Mussel sampling

On the day previous to an assay mussels were collected from the transplantation cages located in the Fal Estuary, packed in cool boxes and transferred to the laboratory within three hours. Here they were cleaned of epibiotic growth and kept in clean filtered sea water at 5°C with continuous aeration in a flow-through tank, in separate compartments, one for each creek. Mussels from the Exe were used as controls and kept under the same conditions. The assays were conducted unsuccessfully in June 1994, and successfully in July 1994 and one year after transplantation in May 1995. Local mussels from Whitsand near Plymouth were used prior to the actual experiments for familiarisation of the technique.

Blood extraction

Blood was collected from the large posterior adductor muscle of the live animals. The mussel valves were prized apart using a sharp strong implement just enough to allow insertion of a hypodermic syringe which had previously been filled with 0.5 ml physiological saline (for composition see Table 7.1) into the muscle tissue. A 0.5 ml sample of blood was drawn into the syringe, ensuring no bubbles entered. The needle was removed from the syringe to prevent possible damage to the cells as the syringe contents were expelled into 2 ml eppendorf tubes, siliconised (sigmacote-coated 24 hours earlier) to prevent blood cells sticking to the container walls. These were stored in a fridge at 5^{O}C before use. Three replicates per site were completed on each date. Samples were checked to ensure appropriate cells had been obtained before starting the assay.

Physiological saline

20 mM HEPES buffer	(4.77g)
436 mM NaCl	(25.48g)
53 mM MgSO4	(13.06g)
10 mM KCl	(0.75g)
10 mM CaCl2	(1.47g)

Table 7.1 Physiological saline: All values per litre H_2O. The solution was gassed for ten minutes (95% O_2 and 5% CO_2) and adjusted to pH 7.3 with 1 M NaOH.

(It was stored in the fridge but used at room temperature).

Neutral Red Probe

20 mg neutral red powder (C.I.50040, Sigma) was dissolved in 1 ml DMSO (dimethyl sulfoxide) in water and filtered through a 0.5 μm millopore cartridge filter attached to an hypodermic syringe. This stock solution was stored at 4°C in a darkened glass container due to its photosensitivity. The working solution was made fresh for each assay by

dissolving 5 µl of this stock neutral red in 995 µl physiological saline, and also stored in a darkened container.

Slide preparation

Microscope slides were washed in methanol and coated with a smear of 1 µl poly-l-lysine (Sigma), diluted with 10 parts water and allowed to air dry. This encourages the blood cells to adhere to the slide by altering the charge of the cell coat.

Neutral red retention assay

The haemolymph samples were agitated to mix up the blood cells and 40 µl were deposited on a prepared slide. These were left to adhere for 15 minutes. Excess haemolymph was tipped off and the slide was washed three times with 40 µl physiological saline. 40 µl saline was added to the attached blood cells, then 40 µl neutral red working solution, leaving a final dye concentration that can be seen under the microscope but is as weak as possible to cause minimum stress to the cells and lysosomes. The slides were placed in a darkened humidity chamber at room temperaure to prevent crystallisation and light reaction.

Slides were checked to ensure this stage of the assay had been successful, *i.e.* that dye had been taken up into the lysosomes. Slides were systematically removed at 15 minute intervals and examined under a microscope (x 500 magnification) for evidence of leakage of dye from lysosomal compartments into the cytosol. This was recorded for individual mussels from the five creeks and the Exe. The experiments were terminated after 180 minutes (Lowe *et al.* 1992, 1995a and b). The time when 50% of cells exhibited dye leakage (assessed quantitatively) from the lysosomes to the cytosol was recorded as 'mean retention

time'. Care was taken to ensure the chambers were kept adequately humid by regularly spraying the tissue lining to prevent crystallisation. Assays were conducted using 'blind' samples.

Nephtys hombergi

This assay was also attempted using the polychaete *Nephtys hombergi*, with magnesium chloride and ms222 used as an anaesthetic. However, blood collection proved too problemmatical and so the experiment was aborted as it was not in the scope of this project.

Statistical analysis

Differences between stations were examined using a two-way ANOVA and Scheffe pairwise comparison test. A correlation analysis of data against metal levels of sediment and mussels themselves was undertaken in Chapter 8.

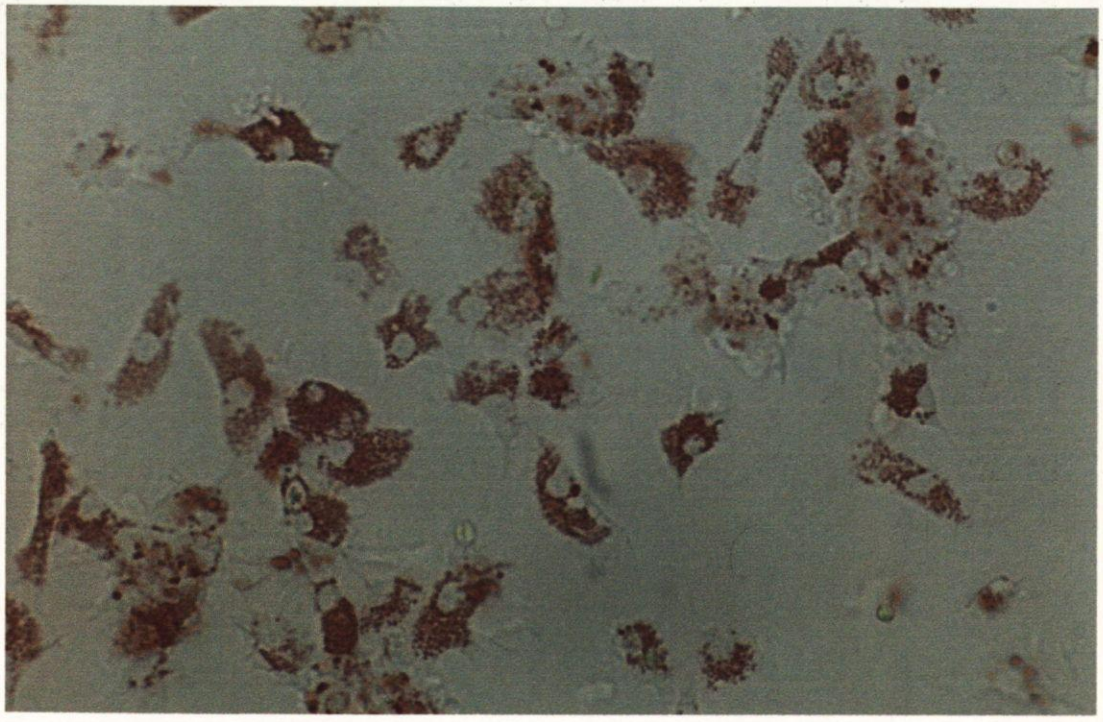
RESULTS

Visual examination of haemocytes.

This detected two cell types. Hyaline cells were spherical in shape and contained few or no lysosomes. Granulocytic cells are irregular in shape and contained numerous lysosomes. The latter type of haemocyte was examined for the purpose of this experiment for lysosomal responses. Haemocytes displaying little or no stress were observed to be irregular in shape and the majority of lysosomes within the cells would be small, spherical and uniform in size. The cells would often elongate in shape an adopt an 'hourglass' shape, being linked by

cytoplasmic strands. In contrast, stressed haemocytes were smaller and rounder in shape and an overall increase in size of the lysosomes were observed. In extreme cases, the dye was observed to immediately enter the cell and be distributed through the remaining cytosol, as well as lysosomes, which indicates severe membrane damage. Figure 7.3 shows the differences observed between healthy and stressed haemocytes.

(a)



(b)

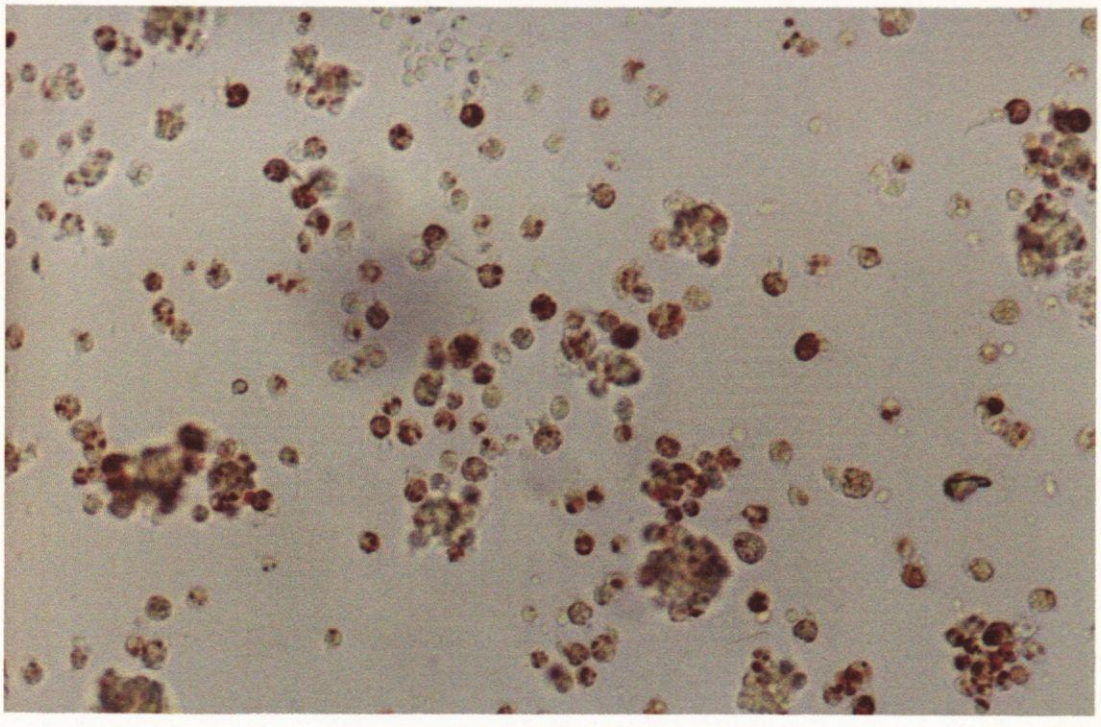


Figure 7.3 *Mytilus edulis*

a) Healthy haemocytes (blood cells) showing irregularly shaped granulocytes and large numbers of small, spherical lysosomes stained by the neutral red retained within them (x 500)

b) Healthy haemocytes (blood cells) showing the altered shape and size of the granulocytic cells and the distribution of the neutral red diffused throughout cytosol (x 500).

Sites	Replicate 1	Replicate 2	Replicate 3	Mean
Restronguet	21	15	0	**12 ± 10.8**
Mylor	98	50	38	**62 ± 31.7**
Pill	79	45	21	**48 ± 29.4**
St. Just	33	45	54	**44 ± 10.5**
Percuil	95	75	66	**78 ± 14.8**
Exe control	140	120	100	**120 ± 20.0**

Table 7.2 50% retention times (minutes) for neutral red dye in *M. edulis* lysosomes. Experiment 1, 13/7/94. (Mean ± S.D.).

Sites	Replicate 1	Replicate 2	Replicate 3	Mean
Restronguet	22	10	26	**19 ± 8.3**
Mylor	64	92	64	**73 ± 16.1**
Pill	67	45	55	**56 ± 11.0**
St. Just	32	74	28	**45 ± 25.5**
Percuil	85	47	92	**75 ± 24.2**
KingHarryFer.	48	54	78	**60 ± 12.0**
Exe control	157	124	107	**130 ± 25.4**

Table 7.3 50% retention times (minutes) for neutral red dye in *M. edulis* lysosomes. Experiment 2, 23/5/95. (Mean ± S.D.).

Results are presented in Table 7.2, 7.3 and Figure 7.4. The neutral red retention assay indicated that mussels exposed to contaminants exhibited probe loss from the lysosomal compartment to the remainder of the cytosol significantly ($p < 1\%$) quicker (between 10 and 98 minutes) than the control mussels which retained the probe for up to 157 minutes.

The assay showed the shortest retention times were in blood cells from Restronguet in May 1995 (12 minutes). Restronguet mussels differ significantly ($p < 1\%$) from those of Mylor and Percuil. At no time was the established heavy metal gradient reflected (see Chapter 8) and the order of retention times and therefore decreasing stress was R > J > P > M > E > control.

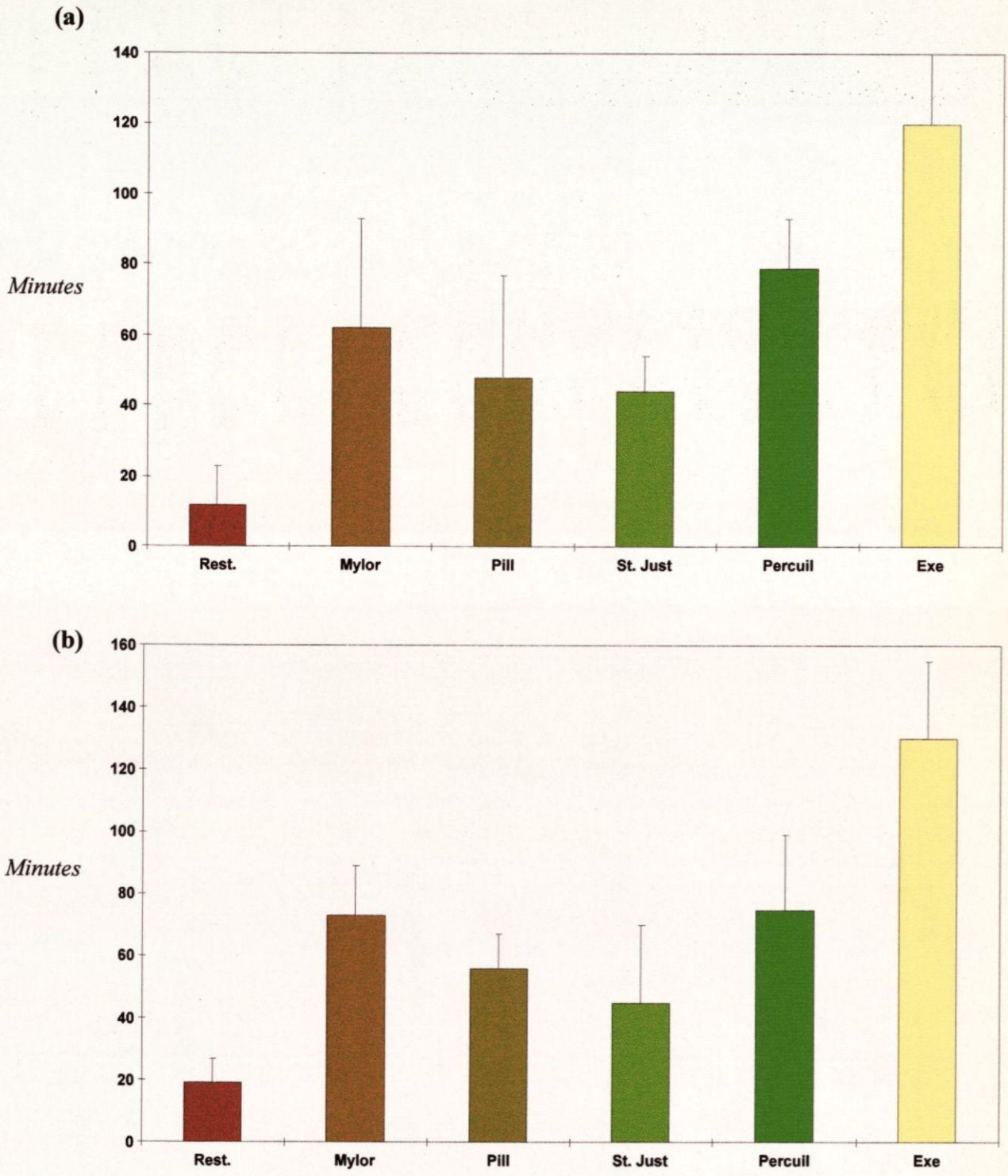


Figure 7.4. *Mytilus edulis* from Fal Estuary creeks:

Neutral red retention times (minutes) for (a) Experiment one (b) Experiment two (Bars denote standard deviation).

CONCLUSION

Summary

The objective of this study was to evaluate this neutral red retention (NRR) assay as a biological impact assessment approach at the cellular level of biological organisation. The results of this *in vitro* technique did not accurately reflect the trend of the established heavy metal gradient in the sediment of the Fal estuary, but did detect differences between sites, especially between mussels transplanted to the Fal and clean Exe mussels. This implies either a low heavy metal detection threshold, *i.e.* the technique is sensitive within certain limitations, or some underlying pollution or stressor in the Fal creeks, *i.e.* there are unidentified contaminants e.g. PCBs, PAHs, as well as the heavy metals present, but this is not known.

These assays confirmed that lysosomes are a target for the toxic action of xenobiotics, as reported by previous studies in fish (Hinton 1989, Kohler 1991, Lowe *et al.* 1992) and mussels (Lowe *et al.* 1995a and b). Literature, however, is conflicting; Mercury and cobalt correlated with retention times in mussels from the Venice lagoon (Lowe *et al.* 1995a), but other metals (iron, manganese, copper and nickel) were not implicated. In contrast, Cheng (1990) found oyster haemocytes did suffer from copper exposure with inhibited lysosomal enzyme release, but not from cadmium exposure. Also, other studies showed copper effects a response (Pipe & Coles 1995). Results of this study indicate a correlation with copper and zinc but none with manganese or iron (see Chapter 8).

Other observations in the assay

It was observed in the stressed samples that as the assay progressed the size of the lysosomes decreases, their number declines, their colour increases in intensity and their shape becomes increasingly round. The presence of enlarged lysosomes (Figure 7.3) from the contaminated mussels indicate another feature of the complex lysosomal response.

Ecological relevance and implementation in monitoring programmes

Haemocytes of bivalve molluscs play an active role in detoxification of heavy metals, *i.e.* in the uptake, distribution to various tissues, and in intra-lysosomal storage. They also have an important role in physiological functions such as defence against invasion by pathogens, wound and shell repair, and nutrient transport (Dean 1977). Consequently the toxic effects of heavy metals in these cells could potentialy affect the survival of the whole organism.

Cajaraville *et al.* (1995) found that numerical changes in the lysosomes of oocytes were significantly correlated with the degree of gonad development and with ecological processes such as general health and growth of mussels. They suggested that lysosomal responses to pollutants might be related to further changes in these processes. To their knowledge there had been no attempts to statistically correlate lysosomal changes with alterations in indicators of population, community structure and function, and stated that this was an important gap to be filled before lysosomal changes can be used as biomarkers of pollution. This present study attempts to go someway towards rectifying that situation, and a correlation of these results with other techniques is presented in Chapter 8 to establish relationships with other levels of biological organisation.

Alterations of lysosomes interfere with the intracellular digestion of food, the normal turnover of proteins and organelles, and the regulation of fusion processes associated with the lysosomal-vacuolar system (Regoli 1992). Therefore, lysosomal responses could be a useful tool for biomonitoring studies as an early warning system for detecting environmental disturbance (Regoli 1992). Such cellular responses to pollutant induced damage are claimed to anticipate impairment at higher levels of biological organisation (Cajaraville *et al.* 1995), and provide rapid and highly sensitive indicators of environmental impact (Moore 1988). The data could be used to deduce that the health of the mussels, and therefore the ability for successful reproduction, will be ultimately affected. Pollutants that affect the viability of haemocytes or interfere with their internal defense functions which are regarded as the primary means of defense of molluscs against invasion by pathogens, may have a profound effect on long-term survival of mussel populations (Cheng 1990). Further studies need to be made of the future reproductive success of mussel populations to determine such long-term trends.

Toxicity of the neutral red dye

Neutral red, a weak base, adds another stress to an already, perhaps, damaged membrane or otherwise healthy cell (Lowe *et al.* 1995b). Such weak bases have been shown to induce structural changes in lysosomes including swelling and vacuolation which result in enhanced autophagy and membrane breakdown (Robbins *et al.* 1984). It produces changes in the fine structure of rat lysosomes as well as lysosomal enlargement and vacuolation (McDonald & Koenig 1965, Ohkuma & Poole 1981). Its use in these studies represents an additional stress to the cells, with the resulting recorded retention time being an integrated response to

membrane damage induced by the contaminant in question and to the neutral red marker probe itself.

Other possible stressors

The June 1994 experiment was cancelled because the mussels were spawning which results in severe lysosomal disturbance. This is potentially significant in decisions regarding whether to use this or any other lysosomal technique in environmental impact studies. It was often found that simply bringing the mussels into the laboratory induced them to spawn, not only in spring, and therefore this questions the suitability of this technique for long-term monitoring studies.

Seasonal differences have been observed; Regoli (1992) found the disfunction of the lysosomal system in mussels from polluted sites were masked by effects resulting from seasonal differences in environmental conditions, such as temperature. This may be the case in the Fal. He also found a large variation in lysosomal membrane stability in the summer months in clean sites, which he attributed to thermal stress induced by the increase in temperature in relatively closed and shallow waters, which again may apply to the Fal creeks. This suggests that mussels are more susceptible to stress during summer than winter, as Moore (1976) also inferred, and that this results in a possible seasonal difference in lysosomal activity. Bayne *et al.* (1978) described similar events leading to severe lysosmal disturbance.

Lysosomal responses have also been linked to salinity and food availability (Cajaraville *et al.* 1995, Moore 1976, Stickle *et al.* 1985). Biotic factors such as size, age and nutritional

state may also affect neutral red dye uptake. Therfore, the use of these responses as biomarkers does not allow diagnosis of the nature of the stressor.

Critique of the technique

The cost-effectiveness and rapidity of this assay is highlighted by Cajaraville *et al.* (1995) stating that they are low in comparison with other chemical or biochemical analyses. The use of an automated system is favoured, but comparable results can be obtained by the simple counting method used in this present assay, which does not require specialised equipment or computer programmes. However, counting by eye is semi-subjective, giving less reproducible results than could be acquired by more sophisticated techniques. It was very difficult to maintain systematic observations every 15 minutes. Computer-assisted image analysis would greatly improve the precision and objectivity of the measurements and would be much less time consuming.

The potential of the assay lies in the fact that it can shed light on the molecular and subcellular mechanisms of pathological alteration induced by contaminants, it can be applied to small tissue samples, and a variety of assays can be applied to the same organism. It also has potential for quantification by image analysis or assessment using a ranked series of photomicrographs (Moore 1988), and the recent advances in use of fluorescent molecular probes offers greater potential.

CONCLUSION

The effects of heavy metal contaminants along a pollution gradient were determined in the common mussel, *Mytilus edulis*, using an *in vitro* technique on live haemocytes. Results demonstrated that lysosomal membrane integrity was impaired following contaminant exposure in transplanted mussels which resulted in an inability to retain the supravital dye neutral red. But the metal gradient of the field sites was not accurately reflected. In the Fal Estuary, as in all field conditions, many factors can contribute to induce stress in sentinel organisms. Due to the existence of factors other than pollution which might induce environmental stress, these 'biomarkers' can only be fully exploited in combination with other biomarkers (Cajaraville *et al.* 1995) and community attributes indicative of exposure to pollutants and other biological effects. The subjective nature of the counting which be improved by image analysis or by using a series of photomicrographs.

CHAPTER 8

Overview & Comparison Of Techniques

Summary of the objectives of this study

The primary objective of this study was to compare, evaluate and validate five biological monitoring techniques at four different levels of biological organisation.

Biological monitoring aims either to predict a pollution incident as early as possible so that it can be forestalled, delayed or reduced, or to assess and monitor its impact retrospectively. To eventually enable prediction it is important to understand the inter-relationships between levels of biological organisation and to evaluate methodology, observing and recording as many cases of pollution as possible. This cannot be done without studying case histories to retrospectively trace what happened and the impact, if any, hence this study concentrating on heavy metal contamination of the Fal Estuary.

This project aimed to investigate critically various biological techniques and correlate the results with input from chemical data, and so suggest which biological techniques are the most pertinent and applicable as gauged by sensitivity, relevance, practicality, speed, ease of use and accuracy. It also aimed to further knowledge of relationships between biological responses at various hierachical levels of biological organisation.

Comparison and evaluation of techniques

The PRIMER program BIOENV (Clarke & Ainsworth 1993) was used to compare quantitatively the relationships between the various techniques and the heavy metal data, as well as additional 'incidental' variables measured during the investigation (dry weight and body condition index (BCI) (Chapter 5), mussel mortality and tissue burden of copper and zinc (Chapter 2).

This analysis involved computing rank correlation coefficients between the elements of the similarity / dissimilarity matrices underlying the three multivariate data sets:

(a) Community structure - a long-term average of the three year study;

(b) Heavy metal concentrations for all metals measured; and

(c) Low level tests (cellular, individual and population) and incidental measures - collectively termed here 'biomarkers'.

Measures for the various biomarkers, which bear different units, were standardised by treating them as an environmental variables matrix which automatically normalises them. All combinations of environmental variables and biomarkers were considered at steadily increasing levels of complexity, *i.e.* k variables at a time ($k = 1,2,3, \ldots, v$) (Clarke & Warwick 1994). The combination of variables which best explain the observed patterns is that giving the highest rank correlation coefficient.

Data were analysed using the program BIOENV in two sets, firstly, heavy metals against biomarkers and secondly, community structure against biomarkers:

Correlation of metal data against 'biomarkers'

The outcome of this analysis is displayed in Table 8.1, which lists the top nine values, and in Appendix 4, which lists the total data set. The best correlation is with three variables, the **copper** and **zinc content** and **dry weight of mussel tissue** ($\rho_W = 0.972$). Of the specific assays of this study (underlined results in Table 8.1) the best correlation is between two variables, the **cellular assay** and **copper in mussels** ($\rho_W = 0.900$), and the three variables of the **cellular assay, mussel respiration** and **zinc content of mussels** ($\rho_W = 0.941$). The single biomarker which is best correlated to the metals is the **cellular assay**, but this has a lower value ($\rho_W = 0.500$) indicating that it is a less reliable biomarker, although its significance was increased (to 0.941) when combined with both mussel respiration rate and zinc levels of mussel tissue. The SfG and population techniques show no correlation with metal levels (see Appendix 4).

k	1	2	3
Best variable combination ρw	Mussel mortality 0.847	Copper in mussels, mussel dry wt. 0.951	**Copper in mussel, mussel dry wt., zinc in mussels 0.972**
	Zinc in mussels 0.797	Mussel mortality, mussel respiration 0.950	Copper in mussels, mussel mortality, BCI. 0.960
	Mussel respiration 0.759	Copper in mussels, zinc in mussels 0.941	Mussel mortality, mussel dry wt., zinc in mussels 0.950
	Mussel dry wt. 0.710	Copper in mussels, mussel respiration 0.932	Copper in mussels, mussel mortality, mussel dry wt. 0.947
	Copper in mussels 0.625	Mussel mortality, zinc in mussels 0.905	Cellular assay A, mussel respiration, zinc in mussels 0.941
	Mussel condition I. 0.549	Cellular assay A, copper in mussels 0.900	Larval assay, copper in mussels, mussel respiration 0.927
	Cellular assay A 0.500	Copper in mussels, BCI. 0.888	Cellular assay A, mussel mortality, mussel respiration 0.927
	Mussel mortality 0.499	Larval assay, copper in mussels 0.880	Copper in mussels, mussel dry wt., mussel respiration 0.925
	Cellular assay B 0.281	Mussel dry wt., copper in mussels 0.876	Mussel mortality, mussel dry weight, respiration 0.924

Table 8.1 Fal Estuary heavy metal contamination.

Combinations of the 15 biotic factors measured, taken k at a time, yielding the best matches of metals and biomarker similarity matrices for each k, as measured by weighted Spearman rank correlation ρ_W; bold type indicates overall optimum. (Underlining indicates actual assays tested). Complete data set in Appendix 4.

Correlation of community structure against other 'biomarkers'

Justification for this analysis.

By definition, pollution ('the damaging effects contaminants have on the environment' Clarke, 1992) is more appropriately measured in terms of biological responses. The four assays at population, individual and cellular levels were compared to the community level, the highest level investigated, and the level closest to the real environmental conditions *i.e.* what the lower level assays should essentially be striving to 'predict' or reflect. Hawkins *et al.* (1994) state that 'bioindicators at lower levels of organisation correlate more directly with environmental levels of a known stress than do those at higher levels'. But higher levels, *i.e.* the community, show the true environmental condition (as illustrated by the 'traditional' BIOENV comparison relating community structure to natuaral environmental variables and metals in Chapter 3) and therefore the lower levels are best evaluated by correlating the biomarkers to the community structure.

The outcome of this analysis is displayed in Table 8.2 and Appendix 4. The long-term community structure best correlated with two variables - the **larval assay** at the individual level of biological organisation and the observed **mortality of mussels**, *Mytilus edulis*. Values are generally lower than with the metals correlation. The best matched single monitoring approach is the **larval assay** (ρ_W = 0.744). Both cellular assays have a much lower match (0.573 for assay B and 0.535 for A) while the SfG technique again shows no correlation (Appendix 4) though its respiration rate component does if combined with the larval assay and cellular assay B (0.637). Population analysis has no correlation (Appendix 4) unless combined with the larval assay and mussel mortality (0.638).

k	1	2	3
Best variable combinations ρ_W	Larval assay 0.744	**Larval assay, mussel mortality 0.779**	Larval assay, cellular assay B, copper in mussels 0.676
	Cellular assay B 0.573	Larval assay, cellular assay B 0.624	Larval assay, mussel mortality, *Nephtys* density 0.638
	Cellular assay A 0.535	Cellular assay B, zinc in mussels 0.612	Larval assay, cellular assay B, mussel dry wt. 0.637
	Copper in mussels 0.529	Larval assay, mussel dry wt. 0.593	Larval assay, cellular assay B, BCI 0.637
	Mussel mortality 0.464	Cellular assay B, mussel dry wt. 0.592	Larval assay, cellular assay B, mussel respiration 0.637
	Mussel dry weight 0.104	Cellular assay B, mussel mortality 0.589	Larval assay, cellular assay B, zinc in mussels 0.637
	BCI 0.028	Cellular assay B, copper in mussels 0.585	Cellular assay B, mussel mortality, mussel dry wt. 0.629
	Zinc in mussels 0.017	Cellular assay B, BCI. 0.569	Cellular assay B, mussel mortality, BCI 0.629
	Mussel respiration -0.065	Cellular assay B, copper in mussels 0.532	Cellular assay A, Cellular assay B, zinc in mussels 0.612

Table 8.2 Fal Estuary long-term macrofaunal community.

Combinations of the 15 biotic factors measured, taken k at a time, yielding the best matches of community and biomarker similarity matrices for each k, as measured by weighted Spearman rank correlation ρ_W; bold type indicates overall optimum. (Underlining indicates actual assays tested). Complete data set in Appendix 4.

Standard correlation analysis between all techniques (minus community level approach) and incidental measurements: inter-level relationships.

In addition to the BIOENV analyses another correlation study was undertaken using a standard correlation technique. This was undertaken to determine relationships between the techniques from the various levels tested and the results are presented in Table 8.3 (all assays and accessory measurements) and Table 8.4 (for assays alone).

	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p
a		.418	.194	.400	*.195*	.804	*.032*	*.500*	*.474*	.543	**.986**	**.890**	**.891**	.207	*.117*	.133
b			**.935**	**.964**	*.915*	*.060*	*.843*	.571	.596	*.474*	.278	*.036*	.034	*.705*	.536	*.165*
c				**.969**	*.863*	*.163*	*.795*	.695	.704	*.581*	.059	*.252*	*.247*	*.699*	*.765*	*.369*
d					*.817*	.041	*.720*	.532	.552	*.394*	.277	*.046*	*.041*	*.577*	*.715*	*.380*
e						.362	*.979*	*.724*	*.733*	.716	*.034*	.219	.218	**.905**	*.358*	*.122*
f							.530	*.815*	*.802*	**.890**	**.880**	**.897**	**.900**	.721	*.073*	*.157*
g								*.809*	*.818*	**.818**	.128	.361	.361	**.969**	*.299*	*.171*
h									*.977*	*.968*	*.627*	*.828*	*.827*	*.897*	.545	*.182*
i										*.957*	*.602*	*.813*	*.813*	*.899*	.545	*.194*
j											.671	**.813**	**.813**	**.927**	*.355*	*.046*
k												**.942**	**.943**	.361	*.172*	.108
l													**.999**	.555	*.422*	.278
m														.556	*.414*	.272
n															*.269*	*.185*
o																*.849*
p																

Table 8.3 Analysis of correlations between the various techniques and biomarkers.

At the 0.1% significance level the value of the correlation coefficient is 0.725; indicated by bold figures. Italics indicate negative values.

a	Scope for growth (SfG)
b	Larval assay
c	Cellular assay A
d	Cellular assay B
e	Copper in mussels
f	Mussel mortality May 1995
g	Mussel mortality June 1995
h	Mussel dry weight
i	Mussel body condition index
j	Mussel respiration rate
k	Mussel absorption rate
l	Mussel consumption rate
m	Mussel clearance rate
n	Zinc in mussel tissue
o	*Nephtys hombergi* density
p	*N. hombergi* mean width

N.B. j, k, l and m are the component physiological measures of SfG.

	Larval assay	Cell assay A	Cell assay B	Worm nos.	Worm wdth.
SfG	0.418	0.194	0.400	-0.117	0.133
Larval assay		**0.935**	**0.964**	0.536	-0.165
Cell assay A			**0.969**	**0.765**	-0.369
Cell assay B				**-0.715**	-0.380
Worm nos.					**-0.849**

Table 8.4 Correlation matrix between the techniques investigated (with the exception of the community level).

Correlations might be expected between some of the different techniques measured *e.g.* at the individual level, a reduced SfG for mussels should result in reduced growth while reduced larval viability would lead to reduced reproductive success; these would result in changes in population structure unless affected by recruitment from outside the area. However, in this study there was no significant correlation between SfG values and larval survival (0.418), nor with the cellular assay at the lowest level of organisation (0.194), nor with the population study of *Nephtys hombergi* at the higher level (0.133). The cellular assays did correlate highly with the larval assay (0.935 and 0.964) and with density of *N. hombergii* (0.765), but not with SfG (0.194 and 0.400).

Some interesting results in the matrix are of note such as the low positive correlation between the cellular assay and SfG (0.194). These two low level biomarkers would be expected to be correlated to one another. Also there is a low negative correlation between copper content in mussel tissue and SfG (-0.195) indicating that this 'biomarker' bears no relationship to the bioavailable metals, while the high positive correlation between the

mussel mortality at one date and the larval assay (0.843) confirms the environmental relevance of this 'biomarker'.

Relevance of the five monitoring techniques

The BIOENV results show that correlation values against the metals were high between some selected assays and incidental measurements. The closest single indicator of the state of the environmental metal gradient was **mussel mortality**; this simple measurement was a better indicator in terms of sensitivity and relevance to the contamination gradient than the more complex ecotoxicological assays investigated. Of these more complex techniques the **cellular assay** had the best correlation with metal gradient, but only at lower value of ρ_W = 0.500 (or ρ_W = 0.941 when combined with mussel respiration and zinc burden). However, if the community itself is correlated against biomarkers, the **larval** and **cellular assays** have the closest association to the faunistic structure.

A question as to whether it is more appropriate for the 'biomarkers' to be closely associated to the metal levels or to the community structure pattern is pertinent as most ecotoxicological assays aim to closely reflect contaminant levels, assuming that they will directly indicate the environmental condition. Rather, low level tests should indicate the state of the highest measurable level of organisation as this is the focus of concern, instead of the concentration of the heavy metals themselves, and this was demonstrated well by the larval survival test.

Are the various techniques at the different levels of biological organisation 'linked' ?

In this investigation there were apparent relationships between cellular and individual levels (larval survival, but not SfG) which could suggest a direct 'link' resulting in cellular

changes impinging on the survival of the organisms. But there was none evident between individual levels and population levels, nor between population and community levels. However, there were relationships between the extremes at the cellular and community levels, and between the individual (larval survival) and community levels. Therfore it appears that any 'linkage' in the investigations of growth between the levels of the individual (SfG) and the population (*N. hombergi* size structure analysis) broke down.

However, GESAMP (1995) propose that it is not necessary to have any links between the levels, that each is valid in its own right, and links may not be causal. But to have such links would be more informative and aid prediction of effects at higher levels. Each would provide different information expressing a different aspect of the response to the stress of heavy metals.

Evaluation and assessment of the individual techniques

A critical evaluation of the various techniques is presented and an assessment of the approaches is then discussed with respect to this validation together with their environmental relevance.

Evaluation

To evaluate the techniques a 'weight of evidence' comparison was constructed in the form of a table of advantages and disadvantages (Table 8.5). It includes factors met in the course of this investigation, such as practicallity, as well as the usefulness and relevance of resulting data sets. Each factor is given equal weight to remove some of the subjectivity of this assessment. Many of the points in the table are already well documented in the

literature (*e.g.* Depledge & Hopkin 1995), this study either confirms or rejects some popular views as well as indicating new factors.

	Com. struct.	Popn. size	SfG	Larval assay	Cell assay
ADVANTAGES					
Low capital cost	+	+		+	
Rapid response time (< 24 hours)			+		+
Sensitive	+			+	+
Environmentally relevant in Fal Estuary	+			+	+
Ease of use	+	+	+	+	
Quick technique (< 24 hours)					+
Long-term condition reflected	+	+		+	
Ease of interpretation	+		+	+	+
Results correlate with metals ?	+			+	+
No seasonal constraints	+	+			
Basic lab equipment will suffice	+	+		+	+
Reliability of experimental protocol	+		+	+	
Not destructive of the test organism					+
Quantitative	+	+	+		
Sub total	**+ 11**	**+ 6**	**+ 5**	**+ 9**	**+ 8**
DISADVANTAGES					
Expensive chemicals and equipment			-		-
Time consuming	-	-	-		
Labour-intensive	-	-	-		
Seasonal restraints			-	-	-
Computer software required	-	-	-		
Poor reliability of experiment				-	-
Destructive of the test organisms	-	-	-		
Subjective (semi)				-	-
Reflects only a 'snapshot' of time			-		-
Complex methodology			-		-
Need vast number of organisms		-			
No correlation to other levels of study		-	-		
Sub total	**- 4**	**- 6**	**- 9**	**- 3**	**- 6**
TOTAL	**+ 7**	**0**	**- 4**	**+ 6**	**+ 2**

Table 8.5 A comparison of advantages and disadvantages of the five techniques under investigation.

This evaluation indicates that the techniques have the following sequence;

community > larval assay > cellular assay > population analysis > SfG.

On the basis of factors such as practicality, cost-effectiveness, relevance and sensitivity, the **community structure analysis** and **larval survival** techniques are the best approaches to use in the estuarine environment of the Fal. The cellular damage assay was fair, but the population and SfG assays were poor.

Assessment

Larval (Chapter 6) and, to a lesser extent, **cellular assays** (Chapter 7) correlated more closely to the community *i.e.* reasonably accurately estimating the situation at higher biological levels. However, neither reflected accurately the persistent metal contamination gradient. Both were sensitive at the exceptionally high levels of metal contamination of the Fal, responsive, semi-quantitative (only by eye), and have a short experimental time. The cellular assay has a short response time (in the order of hours or days) but the larval test reflects more the long-term effects of pollution. Whether this technique can detect pollution in its incipient stages was beyond the scope of this investigation since the mussels were exposed for a period of months. Mussels showed an integrated response to the total pollutant load and were ecologically relevant to the higher level of the community, but did not exhibit any relation to growth (SfG) or population (mean polychaete size). They were measured easily and cost effectively, but an important drawback was their spawning condition which resulted in an inability to perform experiments at particular times of the year. Also variability was high. The larval assay is relevant to population levels as it reflects the reproductive potential of the population but this may be compensated for, or against, by outside recruitment under field conditions.

The **population size structure** analysis (Chapter 4) and **scope for growth** (Chapter 5) tests poorly correlated to other tests, at both higher and lower levels as well as to the metal levels, and thus were unsuitable for testing such a contaminated estuarine environment as the Fal system. They were also time consuming, especially the population assay. The typically high suspended matter content of an estuary could affect the mussel functioning, rendering the SfG test less applicable. However, of all the components of the energy budget, respiration rate did correlate to the metal gradient. It was surprising that the SfG assay, overall, was so unsuccessful in this investigation as this popular approach has been applied successfully to many situations globally for a decade or more, as explained in Chapter 5. Measurements of SfG are necessarily made on live, functioning organisms, *i.e.* survivors, which have effectively been selected by their particular environmental circumstances and therefore the question arises, 'are these assays a true reflection of the total population or just the healthy subset?'

The multivariate analyses of the **community** level approach (Chapter 3) were, perhaps due to its very nature, the most ecologically relevant as well as correlating most closely to the gradient of metal levels (zinc and copper). Despite the drawback of its' labour intensiveness it was, therefore, the best method to detect the effects of heavy metal contamination in the Fal Estuary. Communities changed on a yearly basis and the results of this method responded on that time scale, as an integrated response to the total pollutant.

Implications for monitoring at various levels of biological organisation

Ecological relevance

For a monitoring method to have ecological relevance, the observed responses should parallel changes in growth, reproduction and survival of individuals, populations and ultimately affect the structure of the community. In this respect the community is the most ecologically relevant. Population level tests were ineffective in producing data that could be interpreted, neither size nor density of worms correlating to community or individual levels. Individual larval level and, to a lesser degree, cellular level assays were correlated to community structure and so could be considered to be ecologically relevant, as shown by BIOENV. However, in terms of correlations, no single technique was closely related to metal contamination levels, with the exception of community structure which was best explained by sediment zinc and copper concentrations. Thus the order of suitability of monitoring techniques was **community > larval > cellular > population > SfG.**

Relative sensitivity

For good sensitivity the measured responses should not only differentiate between the presence or absence of metal contamination (*i.e.* between the Fal Estuary creeks and the Exe control site) but also correlate to the observed heavy metal gradient, distinguishing between the particular metal levels associated with each of the five creeks. The larval and cellular assays did not show significant differences between all creeks, whereas the community approach did effectively distinguish distinct macrofaunal assemblages between the creeks, even at taxonomic levels higher than species. The assertion that SfG is consistently more sensitive than the larval assays (Widdows & Salkeld 1992) was not

concentration of contaminant (P) come from chemists who detect chemicals at biologically significant levels but may not know which ones are relevant. Input for ecosystem damage (D) comes from biologists, but this can be too complex unless reduced to a small number or single species monitoring. Also, biological monitoring can be complicated by other variables such as climate, seasons, organic matter, salinity, etc., which themselves exert multiple effects on ecosystems.

Because the Fal environment is so complex and dynamic with respect to the long-term metal tolerance of some species, coupled with the usual competition and predation etc. typical of any instance, it seems impossible to be truly predictive. The only way is to try and encompass all these and not reduce to a single test. Also, many pollution incidents are unexpected, unpredictable and happen very quickly. The value of biological impact assessments in these situations therefore lies in monitoring recovery from pollution or establishing severity of impact. The validity of the term 'early warning' is thus debatable. Every water body would need continuous monitoring just in case of a pollution incident.

If low level tests are to be predictive, forecasting an effect before it manifests itself at a higher level, their validity is not dependent on correlation with the contemporary ecological community condition. In this study the early warning issue has not been addressed because heavy metal contaminant levels are stable and persistent (see Chapter 2 & 3). It would require a study of the community and populations subsequent to this investigation to see if, for instance, the SfG level approach (which bore no relation to current metal levels nor community structure) was truly indicative of an imminent change at higher levels. A treatment plant started operation at the end of 1995 cleaning metals from the water of the Carnon river meaning contamination levels are likely to subside. It would be of interest to

investigate whether any of the low level techniques could predict the consequences of this event.

GESAMP (1995) stated that we should not necessarily expect the results of assays at one level to be able to be predictive of another. But the question arises as to what the relevance of a technique is, or its accuracy, if it cannot be related to the appropriate levels at the focus of concern.

Single or integrated approach ?

Does the use of more than one technique give greater information? Much literature suggests that a suite of techniques at various levels of biological organisation be deployed, *e.g.* the tiered structure approach (GESAMP report No. 55), or integrated approach (Stebbing *et al.* 1992, Depledge & Hopkin 1995). This may be appropriate in some instances but it appears little was gained in the present study from tests at more than one level. It maybe unnecessary to monitor at levels which are irrelevant when it is just as easy and cost effective to monitor at the higher level which is much more informative and accurate.

What is acceptable and what is unacceptable change?

Ecotoxicological development of biomarkers has been, like the development of chemical analytical techniques, both a blessing and a curse. Chemical and biological responses can be measured at increasingly higher sensitivities but this may not be expressed at the level of the population or the community and may cause unnecessary concern to the public and to environmental managers (Chapman 1995). Some effect on the marine environment may even be acceptable (Segar & Stemman 1986).

It is difficult to decide when an effect is truly adverse and hence to decide when it warrants remedial action (Forbes & Forbes 1994, Depledge & Hopkin 1995). It is more obvious with assays like mussel mortality. But what value from the neutral red assay should elicit action? In the SfG assay, Mylor was apparently clean and yet 80% of the mussels died. The results from this assay did not truly represent the population, so it was not an accurate indication of change, and thus no action would have been initiated if this had been the monitoring technique used. Evermore sensitive techniques may also cause false alarms because at what stage does a measured response actually indicate meaningful stress?

The best level of approach and hence technique to use

Of the techniques investigated **community structure analysis,** *Mytilus edulis* **larval survival** and the **cellular damage assay** were judged to be the best techniques to reflect the heavy metal gradient within creeks of the Fal Estuary. Other studies have also proposed that larval survival is a good indicator of pollution: Nipper *et al.* (1996) found that estuarine amphipod mortality assays best correlated with ecological effects and that growth measures were poorly sensitive. However, in contrast to the present study, she found that benthic community tests had no correlation with toxicity tests or to contaminants. Scott Carr *et al.* (1996) also found varying results from toxicity tests (microtox assay / amphipod survival / embryo development of echinoid sea urchin) with amphipod survival the least sensitive and sea urchin embryo development the most sensitive. GEEP found that the oyster larval survival assay was very sensitive (Stebbing *et al.* 1992). This study endorses the view of, for example, Chapman (1995) and Borgman *et al.* (1993) that survival data should be measured as there remain instances and contaminants where such data are more sensitive indicators of toxicity than either growth or reproduction.

The low level larval assay in addition to correlating closely to the community response was quick and relatively inexpensive. Does this advantage suggest that it is adequate and can be used in place of the higher level approach? Chapman (1995) said that it could not do so until it was linked to higher level effects and indicative of early warning, not just of exposure. This study has suggested that they are related to higher level effects (but has not shown a direct physiological reason for a link) but whether it was indicative of early warning and not just of exposure was neither proven or disproven in this investigation.

However, two incidental measurements made during this investigation, **mussel mortality** and **body condition index** (BCI) were even more accurate in reflecting the metal contamination gradient. Using mortality as a toxicity test was a reliable indicator of contamination levels and is an old approach, used since the early half of this century in Europe and North America (Chapman 1995). Typically, fish are put in cages in contaminated streams or estuaries and the time for 50% of them to die (TLM_{50}) estimated. The contamination gradient also correlated significantly with mussel tissue copper and zinc levels suggesting metals are still in the water or suspended solids. Since the 1970s, pollutant residues in aquatic organisms have been recognised as a valuable index of exposure (Philips 1980) and important as it relates to the biologically available amount of contaminant.

The population level approach was a poor indicator of the environmental condition (both metal levels and community structure) and was judged to be unsuitable as a monitoring approach because of its labour intensity, practicality, time and lack of decisive endpoint. The cellular assay related poorly to the metal levels, and the SfG assay even less so and hence these are inadequate techniques to use in this particular situation. In addition, if the cellular damage and SfG assays involve a rapid response as is claimed, they could miss any effect of contamination in the same way that chemical monitoring can (see Figure 1.1, Chapter 1).

In conclusion, an integrated approach involving larval survival and mussel mortality in addition to the highest level of community would give the clearest representation of the condition of the environment. However, if only one level and technique had to be selected as the best approach to use then the highest level, community structure analysis, was most favourable in terms of practicality, sensitivity and relevance, confirming the following statement:

'Biological surveillance of communities....is perhaps the most sensitive tool available for quickly and accurately detecting alterations in aquatic ecosystems'

(Cairns & Pratt 1993).

CHAPTER 9

Conclusions

Advantages and disadvantages of individual techniques

Community structure analysis

At the level of the community, multivariate analysis using the PRIMER program BIOENV, identified significantly different macrofaunal assemblages associated with the various creeks and these correlated best with sediment zinc levels spatially. This pattern was still apparent at higher taxonomic levels. This approach was hence a very good indicator of the metal contamination and of the ecological condition.

Population size structure analysis

At the level of the population, frequency size analysis using the polychaete *Nephtys hombergi* did not produce data that related to the heavy metal pollution of the Fal Estuary. It was impractical as a routine monitoring approach due to the requirement for very large samples. Hence this approach was a poor indicator lacking correlation with both sediment metal levels and community attributes.

Scope for growth

At the level of the individual, bioassays based on physiological energetics of *Mytilus edulis* in terms of SfG did not provide a sensitive and general measure of water quality being poorly correlated to both metals and community structure, although an individual component of the energy budget, respiration rate, did correlate with the contamination gradient.

Larval survival

Again at the level of the individual, *M. edulis* populations transplanted from the Exe to the Fal Estuary did not provide gametes for larval survival. However, larvae reared from unstressed populations responded sensitively to changes in quality of water from the Fal, and results from this method correlated particularly well to community structure. Thus this approach gave a good indication of the state of the ecosystem.

Cellular damage assay

At the level of the cell, the assay of lysosomal cell damage provided a relatively sensitive measure of heavy metal contamination of the Fal Estuary, but the correlations were weak. However, of the single low level tests compared in this study, this was the best indicator of the metal gradient of the system.

All techniques had disadvantages. The community structure assay is time consuming in terms of sorting and identifying organisms; mussels for larval survival assays tended to spawn at inconvenient times in the low level tests; and the population approach was highly labour intensive and produced results that were difficult to interpret. SfG was the least problemmatical assay to conduct but its sensitivity was poor.

Weight of evidence comparison

This comparison of advantages and disadvantages of each techniques highlighted that the most favourable technique was community analysis, followed by larval survival, cellular damage, population analysis, and finally SfG.

Heavy metal gradient

Stress levels indicated by each test did not closely reflect the heavy metal contamination gradient of the Fal Estuary sediments, although community analysis identified different macrofaunal assemblages within each creek sampled, most closely correlated to sediment zinc and copper levels.

BIOENV comparison

For the first time the PRIMER program BIOENV was used to compare and validate the results of tests at differing levels of biological organisation. It showed that the larval assay was most closely correlated to community structure and the cellular assay to metal levels. Community structure was best correlated to zinc and copper levels. The question of relevance to community structure or sediment contamination was discussed.

Relationships between the levels

It is known that individual and cellular levels are sensitive to toxic chemicals but this study has shed light on the meaning of adverse responses in relation to higher levels of biological organisation that have so far been disputed. Low level stress-induced changes in *Mytilus edulis* have been seen to be related to effects at community level of biological organistion (but not to population level) in terms of larval survival and cellular damage.

Incidental measures

Simple incidental measurements made through the course of the study were in fact more closely related to the metal contamination gradient - namely mussel mortality, metal burden body condition index and tissue dry weight - than the actual assays tested in the investigation. In particular, the mortality observations of *Mytilus edulis* are further evidence of the unsatisfactory condition of the Fal Estuary creeks.

Laboratory to field verification

This study has linked results of laboratory assays to field observations, a much-needed verification which has been largely non-existent. It has also correlated assay results with other indicators of contamination, data which until now have been few and far between. The study also validated larval and cellular tests against community structure estimates, which themselves are the most relevant reflection of the condition of the environment.

Further studies

One of the most relevant areas of study requiring further attention is to examine the time of delay between observed effects at the various levels. If the first detectable response is at the molecular and cellular levels how long is it before effects become detectable at other levels. Further investigation is also needed to clarify for how long low level effects are observable.

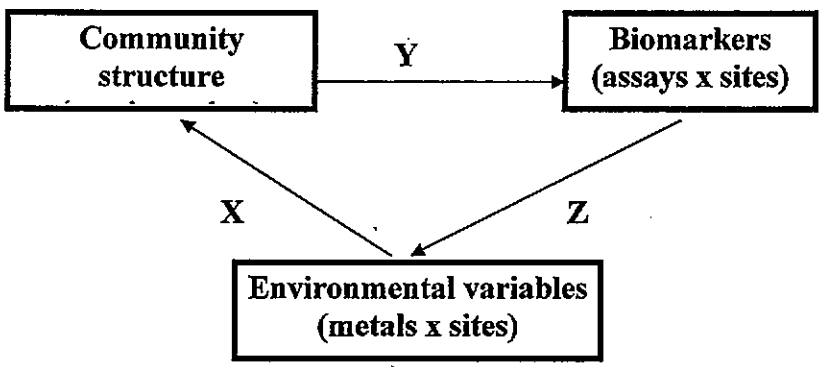
The question of predicting the consequences of a pollution event in the Fal would need further community structure analysis studies. The SfG assay showed that Mylor Creek was cleaner than expected while St. Just and Percuil Creeks were more stressed than anticipated.

Further studies at the population level of biological organisation although recognised as being problemmatical are highly desirable. This would involve larger sample collections as well as, for instance, analyses of fecundity, biomass production and life tables and would contribute to the basis on which predictive modelling could be developed.

Finally, mechanistic examination of the links between the various heirachical levels of biological organisation should ideally be undertaken.

FINAL CONCLUSIONS

BIOENV procedures have been used to examine relationships between three multivariate sets of data, all from the same sites:



The relationship X has been performed before (the traditional use of BIOENV), but this study has for the first time applied the BIOENV approach to Y and Z. These latter two are important if we are to assess the ecological relevance of various biomarker techniques (Y) or the degree to which they reflect the contaminant gradient (Z). It is stressed that the utility of any biomarker approach is best

evaluated by Y rather than Z, hence the importance of this current novel investigation.

This study deployed five different techniques to examine the effects of heavy metal contamination and determine which level of biological organisation of the Fal Estuary system is best suited to indicate its condition. Four levels were assayed to detect stress on individual marine organisms, populations, communities in the field, to determine their value and limitations, and to identify those indicators that reflect the impact of the heavy metal contamination in that environment. Some of the approaches were established techniques, *e.g.* community structure analysis and scope for growth, and some were more recent ecotoxicological methods, *e.g.* the neutral red retention assay. Results were used to produce an experimental validation, evaluation and intercomparison of the selected techniques, and showed that the order of preference was community structure > larval survival > cell damage > scope for growth > population size-frequency analysis.

Appendices

Appendix 1

Sediment heavy metal concentrations 1993

Site		Copper µg/g	Zinc µg/g	Manganese µg/g	Iron mg/g
Restrong.	1	3801.32	8717.67	453.08	91.50
	2	3173.65	4999.50	446.07	70.97
	3	2852.62	4970.96	468.90	67.87
	4	2563.64	4537.44	472.71	67.57
	5	1689.92	2375.27	396.02	37.06
	6	2659.47	3973.13	387.92	70.70
	7	2383.58	3566.56	348.26	134.37
	Mean	**2732.02**	**4734.36**	**424.70**	**77.14**
Mylor	1	1257.91	1639.72	350.89	45.70
	2	1371.93	1747.33	417.19	42.16
	3	1176.88	1553.00	376.73	46.79
	4	1043.03	1530.67	400.20	39.56
	5	914.37	1157.81	177.59	31.63
	Mean	**1152.82**	**1525.70**	**344.52**	**41.16**
Pill	1	611.92	957.47	281.31	45.71
	2	354.29	596.33	192.93	23.22
	3	518.84	751.54	224.94	32.88
	4	472.04	786.72	211.86	33.14
	5	625.51	894.05	255.16	31.63
	Mean	**516.52**	**797.22**	**233.24**	**33.31**
St. Just	1	352.71	587.71	255.56	31.19
	2	368.48	562.26	263.93	31.87
	3	384.35	611.41	245.45	36.46
	4	320.69	527.99	223.74	39.64
	5	399.46	640.29	227.23	38.10
	Mean	**365.13**	**585.93**	**243.18**	**35.45**
Percuil	1	149.44	349.88	224.18	54.67
	2	151.30	309.05	264.40	34.75
	3	161.37	282.05	179.17	31.68
	4	183.18	274.24	205.49	36.70
	5	168.89	256.55	212.21	36.03
	Mean	**162.83**	**294.35**	**217.09**	**38.76**

Sediment heavy metal concentrations 1993 (continued)

Site		Cadmi. µg/g	Cobalt µg/g	Chromi. µg/g	Nickel µg/g	Lead µg/g
Restron.	1	6.93	35.52	32.21	47.58	361.70
	2	3.45	27.46	34.36	40.66	268.62
	3	3.96	26.57	37.69	36.20	173.63
	4	3.95	25.68	36.86	37.66	256.49
	5	4.13	18.76	23.13	28.29	129.23
	6	4.03	26.56	34.81	37.95	228.69
	7	4.83	22.76	40.61	32.16	196.13
	Mean	**3.77**	**26.18**	**34.23**	**37.21**	**188.97**
Mylor	1	3.07	17.23	47.79	39.19	204.97
	2	2.62	16.56	36.81	33.35	202.63
	3	2.61	16.50	31.64	33.95	185.93
	4	2.87	16.49	39.87	35.81	169.15
	5	2.96	16.78	34.98	31.43	150.17
	Mean	**2.82**	**16.71**	**38.21**	**34.74**	**182.57**
Pill	1	2.32	14.33	23.40	31.46	143.57
	2	2.22	11.80	13.38	23.54	88.16
	3	2.00	12.11	21.53	23.74	98.14
	4	2.04	13.10	15.77	26.06	103.95
	5	2.04	12.92	24.48	25.71	130.41
	Mean	**2.12**	**12.85**	**19.71**	**26.10**	**112.84**
St. Just	1	1.78	13.16	25.82	28.42	94.29
	2	1.82	12.48	42.60	31.27	94.14
	3	1.97	13.68	27.12	31.37	100.62
	4	1.62	11.60	25.39	32.09	101.63
	5	1.62	12.24	48.84	28.74	118.68
	Mean	**1.76**	**12.63**	**33.95**	**30.37**	**101.87**
Percuil	1	1.63	12.89	27.18	32.96	98.29
	2	1.41	11.94	27.66	31.27	77.71
	3	1.74	11.52	26.11	27.93	66.86
	4	1.23	21.22	42.81	31.78	164.02
	5	1.32	11.73	53.90	31.36	83.41
	Mean	**1.21**	**13.86**	**35.53**	**31.06**	**98.05**

Sediment heavy metal concentrations 1992

Site		Copper µg/g	Zinc µg/g	Manganese µg/g	Iron mg/g
Restrong.	1	2954.08	6573.85	346.13	56.23
	2	3004.87	7321.65	323.24	57.60
	3	1854.32	4691.37	446.54	75.63
	4	1639.50	4887.83	487.57	62.21
	5	1588.32	5968.60	477.24	57.85
	Mean	**2208.21**	**5888.66**	**416.14**	**61.90**
Mylor	1	1354.11	1899.33	285.65	36.95
	2	1178.69	2008.55	387.98	48.21
	3	942.25	1174.41	465.45	43.22
	4	1114.45	1677.67	478.65	48.98
	5	856.65	1890.98	421.13	31.14
	Mean	**1089.23**	**1730.18**	**377.77**	**41.70**
Pill	1	579.18	994.72	163.54	23.65
	2	678.95	886.92	285.25	34.66
	3	784.62	956.77	256.53	45.25
	4	787.65	686.35	194.65	33.63
	5	596.84	994.98	266.50	38.56
	Mean	**685.44**	**903.94**	**233.29**	**35.15**
St. Just	1	321.32	695.54	355.62	38.99
	2	394.33	836.47	218.59	42.24
	3	563.98	561.18	247.11	31.25
	4	314.98	654.97	154.85	32.22
	5	337.65	586.04	231.45	41.78
	Mean	**386.45**	**605.79**	**241.52**	**27.29**
Percuil	1	213.63	375.21	362.32	46.32
	2	187.84	210.36	187.63	33.85
	3	169.96	274.63	208.22	36.85
	4	183.83	384.22	235.44	38.66
	5	197.32	339.88	161.45	34.51
	Mean	**190.51**	**329.33**	**231.01**	**38.03**

Sediment percentage fines and organic matter 1991 (from Perryman 1991), 1992 and 1993.

Site		1991		1992		1993	
		% o.m.	% fines	% o.m.	% fines	% o.m.	% fines
Restro.	1	6.8	89.2	8.6	90.4	7.6	87.4
	2	6.4	88.6	7.8	86.4	8.4	90.4
	3	5.6	81.0	8.6	90.4	8.8	81.8
	4	5.7	91.6	8.2	94.2	9.2	92.6
	5	3.9	64.6	8.8	92.8	5.8	78.4
	6	8.6	76.6	7.6	76.8	7.2	32.4
	7	6.5	49.6	9.2	60.8	6.8	38.6
	Mean	**6.2**	**77.3**	**8.4**	**84.54**	**7.6**	**71.65**
Mylor	1	8.5	97.7	9.4	98.4	9.8	91.6
	2	8.9	95.5	9.8	96.2	10.4	97.7
	3	-	93.5	10.4	92.4	10.5	97.6
	4	8.4	92.6	10.8	92.8	9.4	92.5
	5	8.1	98.5	10.4	90.8	10.6	91.6
	Mean	**8.4**	**95.56**	**10.16**	**94.12**	**10.14**	**94.2**
Pill	1	8.8	92.0	9.2	97.4	9.2	95.1
	2	9.3	97.7	8.8	90.4	9.8	92.9
	3	9.6	93.0	8.8	92.8	9.2	88.3
	4	9.2	92.5	9.2	99.0	8.8	96.3
	5	8.8	96.1	8.0	92.4	9.8	95.7
	Mean	**9.1**	**94.2**	**8.8**	**94.4**	**9.3**	**93.6**
St.Just	1	11.8	96.3	16.4	96.4	16.2	97.8
	2	10.2	95.1	14.2	95.2	16.9	98.5
	3	20.5	92.9	15.8	92.5	15.8	92.0
	4	10.7	92.0	12.8	94.8	18.2	92.4
	5	10.5	88.9	11.4	90.4	18.4	90.8
	Mean	**12.7**	**93.0**	**14.1**	**93.86**	**17.1**	**94.3**
Percuil	1	10.2	96.0	9.8	96.3	8.4	92.6
	2	9.0	94.3	9.6	92.5	8.8	93.6
	3	7.7	95.7	8.8	97.7	7.8	97.6
	4	8.5	90.7	10.4	93.5	8.4	95.7
	5	7.5	97.7	8.4	98.5	8.8	92.4
	Mean	**8.5**	**94.8**	**9.4**	**95.7**	**8.4**	**94.38**

Pore water salinity and pH November 1993

Site		Salinity	pH
Restronguet	1	28	6.8
	2	29	7
	3	31	7
	4	32	7.1
	5	31	7.1
	6	31	7.1
	7	31	7
	Mean	**30.4**	**7.0**
Mylor	1	30	7
	2	30	6.8
	3	31	6.5
	4	30	6
	5	17	6.8
	Mean	**27.6**	**6.6**
Pill	1	17	6.9
	2	31	6.6
	3	31	6.7
	4	30	6.8
	5	27	6.8
	Mean	**27.8**	**6.7**
St. Just	1	27	6.6
	2	33	6.7
	3	32	6.4
	4	10	7
	5	26	6.2
	Mean	**25.6**	**6.5**
Percuil	1	31	6
	2	31	6.35
	3	30	6.6
	4	30	6.7
	5	31	6.8
	Mean	**30.6**	**6.5**

Mytilus edulis heavy metal concentrations

Site	Replicates	Copper	Zinc	Mangane.	Iron
Restrong.	1	39.00 ±0.27	368.77	7.55	763.72
	2	21.14	329.80	2.46	109.76
	3	-	406.34	3.22	105.74
	4	22.47	-	2.59	112.36
	5	26.00	275.50	3.20	113.50
	Mean	**27.15**	**345.10**	**3.80**	**241.01**
Mylor	1	10.55	140.17	2.51	90.93
	2	4.47	166.46	3.18	93.91
	3	6.12	397.44	2.95	109.69
	4	-	-	-	-
	5	5.45	181.86	3.41	115.46
	Mean	**6.64**	**221.48**	**3.01**	**102.49**
Pill	1	11.11	160.35	2.22	119.07
	2	2.70	142.22	2.16	88.68
	3	4.80	66.77	2.20	82.14
	4	9.96	62.26	2.93	87.66
	5	4.89	55.34	2.20	100.41
	Mean	**6.69**	**97.38**	**2.34**	**95.59**
St. Just	1	2.02	71.21	2.35	77.60
	2	6.87	43.26	2.43	70.61
	3	2.48	61.87	3.75	89.65
	4	2.92	84.16	3.16	76.51
	5	2.04	46.11	2.12	92.74
	Mean	**3.26**	**61.32**	**2.76**	**81.42**
Percuil	1	2.55	115.95	2.50	117.49
	2	1.99	47.84	1.79	66.28
	3	0.99	35.44	1.64	71.88
	4	3.44	31.95	1.81	87.49
	5	1.99	36.44	1.89	64.39
	Mean	**2.19**	**53.52**	**1.92**	**81.50**
Exe	1	1.49	71.07	2.63	95.42
	2	3.44	50.70	2.16	88.12
	3	2.93	40.64	3.72	69.05
	4	2.42	44.53	1.45	58.56
	5	1.95	52.84	1.95	76.82
	Mean	**2.44**	**51.95**	**2.38**	**77.59**

Appendix 2

Notes on organisms present in macrofauna samples.

Abra prismatica - inhabits clean sand and muddy sand, from low water downwards, common.

Anenome indet - few have penetrated into low salinity, some *e.g. Actinia equina* can tolerate low salinity down to 8% as long as the salinity is stable, *Nematostella vectensis* has been found in brackish pools in the Isle of Wight.

Capitella capitata - lives in foul mud where it may be locally very abundant, sedentary, burrow and live in the mud.

Carcinus maenas - resistant to changes in salinity due to its ability to osmo-regulate at salinity lower than the blood concentration, the only crab which penetrates well into estuaries in Britain, a generalised predator eating anything it finds.

Cerastoderma edule - a burrowing (down to 5-6 cms.) bivalve which extends two short papillate siphons just above the surface and take in water by cilliary action to filter off any suspended food material such as small plankton organisms it may contain, most frequent habitat is muddy sands but can also occupy coarse gravels and stiff muds, spawning begins in march and extends through the summer, veligers may remain in plankton for 2 - 3 weeks before settling, may live 8 years.

Glycera capitata - a common worm of the lower sandy shore, mud or muddy sand, errant, carnivorous, burrows in clean or muddy sand

Heteromastus filiformis - eat mud by extrusion and protrusion of the proboscis, near the mouths of estuaries.

Hydrobia ulvae - form an important part of the estuarine fauna in their great numbers, their dispersal is aided by the habit of floating on the underside of the surface of the film of water, during which it secretes a mucus raft which serves as a floatation device (to maintain position on the shore) as well as trapping small particles such as diatoms which are then eaten by the snail, when the tides ebbs the snails detach and sink to the bottom where they crawl and browse on detritus and diatoms, they then burrow under the mud surface before emerging for the next incoming tide. *H. ulvae* prefers the highest salinity of the Hydrobia genus at 10-33 ‰, and is the most abundant on bare exposed areas. The floating habit does not take place in areas of variable vertical salinity, and instead they maintain their

position on the shore by crawling and burrowing. *H. ulvae* feeds on *Ulva*, *Enteromorpha*, blue-green algae and diatoms, and on bare mud flats the food seems to consist mainly of bacteria attached to organic debris. The breeding season extends over some months, February and march as well as in the autumn, egg capsules are attached to the shells and the veligers may spend a short while in the plankton.

Limnopontia - an opistobranch gastropod, can swim with varying efficiency normally at spawning time in spring, a deposit feeder picking up organic matter particles from the sediment surface as it moves across it, a dweller on mud-flats at low-tide spending much of its time out of water and in moist air, particularly in South and West.

Macoma balthica - lives in thick mud, and muddy sand, occurring principally from the upper regions of the inter tidal zone to low water, tolerant of low salinity and particularly common in estuaries, similar in structure to *Scrobicularia* but has different habits, not burrowing so deeply and moving about frequently along U-shaped tracts in search of food, main food derived from digestion of micro-organisms in deposits that are sucked up by the inhalant siphon.

Manayunkia aestuarina - in surface mud of estuaries, thin walled tubes of microprotein.

Melinna palmata - also very common where rotting *Laminaria* mixed with sediment and belts of algal debris.

Nephtys hombergi - frequents all sediments but the finest mud, burrowing but also highly active swimmers, more prolific in cleaner sand than mud, proboscis used in feeding (deposit feeder) as the worm burrows it takes in food in the form of other small animals or detritus, errant, penetrates further into estuaries than other species of *Nephtys*, does not construct a permanent burrow but moves through the sand in search of prey.

Nereis (Hediste) diversicolor - intertidal omnivorous scavenger, making burrows in black muddy sand, very typical of estuaries and tolerant of, in fact thrives in very low salinity (therefore is very common where salinity if interstitial water is lowered by the influence of small streams), to which its physiology is able to adjust, actively maintaining the difference in concentration between their body fluids and slightly saline water around them (osmo-regulation), teeth and strong jaws for burrowing and feeding, spawning occurs at the sea surface in February in the south of England as the water temperature rises above 5 °C and the trochophore larvae remain as plankton for a short while before settling down on the sediment for the benthic phase of their life history. However, the larvae do not enter the plankton and remain on the mud surface or within the parental burrow (such avoidance being seen as an adaptation to estuarine life). Errant, feeding varies from microfauna, such

as copepods, nematodes, foraminifera, to larger prey, such as juvenile fish, also they can filter small particles from suspension with a mucous net at the entrance to its burrow.

Ostrea edulis - basically a marine form with the ability to tolerate brackish water down to 20‰, *Crassostrea* is capable of penetrating further into estuaries than *Ostrea* and of surviving in salty conditions.

Phyllodoce mucosa - swim and crawl, carnivorous but lack jaws and instead use their proboscis by extruding it to catch prey, errant, common on inter tidal sand and mud bottoms

Pygospio elegans - sessile, occupies long flexible tube of fine sand grains embedded in mucus, mid-shore to sub-littoral in mud, burrowing, collects food with two long grooved tentacles from water or sediment surface, in stable brackish waters it can penetrate down to a salinity of 8‰, larvae can become planktonic.

Scrobicularia plana - an inter tidal species inhabiting soft bottoms of clay or mud, with abundant organic detritus, in estuaries, is well adapted to live in deep soft mud with a large foot and long siphons and very active in sucking up deposits from the mud surface and for respiratory purposes, may live as long as 18 years.

Tharyx marioni lives just below the surface of mud full of rotting algae, with its thin respiratory tentacles protruding above the surface, feeding tentacles trap particles of food and convey them to the mouth, sedentary, very common where decomposing *Laminaria* is mixed with the sediment.

Tubificoides benedeni - 'sludge-worms', living in muddy sediments surrounded by decaying organic material and able to withstand high levels of such pollution.

	Filter feeder	**Deposit feeder**	**Herbivore**	**Carnivore**
Mobile epifauna	crustaceans		gastropods	gastropods crustaceans
Sedentary epifauna				coelenterates
Attached epifauna	bivalves polychaetes			
Mobile infauna	polychaetes	bivalves polychaetes oligochaetes		gastropods crustaceans polychaetes
Sedentary infauna	bivalves	polychaetes crustaceans		coelenterates
Attached infauna	bivalves			

Appendix 3

Species abundance data

COMMUNITY DATA NOVEMBER 1992

	R1	R2	R3	R4	R5	R6	R7	M1	M2	M3	M4	M5	P1	P2	P3
Nephtys hombergi	6	13	37	17	23	8	17	43	3	30	49	22	19	16	24
Nereis diversicolor	17	2		1		3	7					3		2	
Streblospio shrubsoli			7		2		4	122	180	25	107	15	18	58	49
Pygospio elegans			10	1	2	2	3		1	1		4			
Spio martinensis			2				3								
Phyllodoce mucosa		1			2	4	4								
Glycera spp.														1	
Tharyx marioni			3		3				3	3		1		2	
Ampharete acutifrons															
Melinna palmata									1		4		1		1
Manayunkia aestuarina								6	5		4	7	2	37	
Paranais littoralis															
Tubificoides benedeni	7	3	27	5	7		4	624	948	179	824	72	19	539	182
Capitella capitata															
Heteromastus filiformis															
Hydrobia ulvae			4					106	242	8	22	10	2	91	194
Cerastoderma edule				1	2		1	1	6	1	4	4	1	24	6
Scrobicularia plana						2	2	2	2	2	2	1	2	3	1
Abra prismatica								1		1					
Macoma balthica														2	
Ostrea edulis												1			
Carcinus maenas											1				
Mysid *Indet*									1	1	2				
Anenome *Indet.*													1		
Amphipoda *Indet.*															
Limopontia depressa															
(A)	18	19	90	25	41	19	45	905	142	251	101	378	238	775	457
(S)	3	4	7	5	7	5	9	8	01	10	98	1	9	1	7
									1			1		1	

	P4	P5	J1	J2	J3	J4	J5	PE1	PE2	PE3	PE4	PE5
Nephtys hombergi	25	28	2			9	4	8	9	6	8	3
Nereis diversicolor			53		5		10					
Streblospio shrubsoli	59	34	240	50	35	30	3	236	888	523	890	534
Pygospio elegans			2	4				6	24	43		
Spio martinensis								1			6	
Phyllodoce mucosa								2			6	
Glycera spp.												
Tharyx marioni		3	10				1	20		28	1	
Ampharete acutifrons											1	
Melinna palmata		1						2	24	6	22	3
Manayunkia aestuarina	6		54	58	2	6	1	2424	304	61	19	2
Paranais littoralis				6								
Tubificoides benedenni	265	225	799	693	76	255	290	118	3424	828	319	51
Capitella capitata			2				213	8				1
Heteromastus filiformis												
Hydrobia ulvae	90	92		12		2	21	2	11	22	2	15
Cerastoderma edule	9	6	4	6		2	3	1	26	4	6	
Scrobicularia plana			8	2			1	2	24	3		3
Abra prismatica		1										
Macoma balthica					2				32			
Ostrea edulis												
Carcinus maenas												
Mysid *Indet*										2	1	
Anenome *Indet.*												1
Amphipoda *Indet.*												
Limopontia depressa												
(A)	454	390	117	83	12	304	547	3905	4847	152	129	121
(S)	5	8	410	8	16	6	10	1	1	61	01	48

Appendix 4

BIOENV results: Long-term community vs. biomarkers

Number of variables	Correlation value (r)	SfG	Larval assay	Cell assay a	Cell assay b	Copper in mussel	Mortality May 95	mortality June 95	Mussel dry wt.	Mussel BCI	Mussel respiration	Mussel absorption	Mussesl consumption	Mussel clearance	Zinc in mussels	*Nephtys* width
1	0.744		*													
1	0.573				*											
1	0.535			*												
1	0.529					*										
1	0.464							*								
1	0.104								*							
1	0.028									*						
1	0.017														*	
1	-0.065										*					
1	-0.228						*									
1	-0.37															*
1	-0.477												*			
1	-0.58											*				
1	-0.581	*														
2	**0.779**		*					*								
2	0.624		*		*											
2	0.612				*										*	
2	0.593		*						*							
2	0.592				*				*							
2	0.589				*			*								
2	0.585				*	*										
2	0.569				*					*						
2	0.532			*		*										
2	0.529		*	*												
2	0.523			*				*								
2	0.518		*							*						
2	0.508			*	*											
2	0.507		*			*										
2	0.495			*											*	
2	0.484		*								*					
2	0.472					*		*								
2	0.469		*												*	
2	0.466		*												*	
2	0.46							*	*							
2	0.46							*		*						
2	0.422							*								*
2	0.419				*						*					
2	0.363							*							*	
2	0.356			*					*							
2	0.356			*						*						
2	0.351							*			*					
2	0.341					*									*	
2	0.322			*							*					
2	0.311							*					*			
2	0.311							*						*		

COMMUNITY DATA NOVEMBER 1993

	R1	R2	R3	R4	R5	R6	R7	M1	M2	M3	M4	M5	P1	P2.	P3
Nephtys hombergi	1	1	10	1	2	8	10	4	29	8	8	3	3		1
Nereis diversicolor	27	9			1		2	1	1						
Streblospio shrubsoli						7	2	36	46	8		36	1	1	10
Pygospio elegans						4		-							2
Spio martinensis										1					
Phyllodoce mucosa															
Glycera spp.															
Tharyx marioni								4	1	2					
Ampharete acutifrons															
Melinna palmata													1		
Manayunkia									4		1				
Pectinaria littoralis															
Tubificoides benedeni	6	9		1	3	4		69	239	202	88	257	12		56
Capitella capitata															
Heteromastus filiformis										2					
Hydrobia ulvae								8	30	27	1	4			3
Cerastoderma edule						2	1	1	2	3	2	2	1		1
Scrobicularia plana									1						
Abra prismatica															
Mecoma balthica									1						
Ostrea edulis															
Carcinus maenas															
Mysid *indet*						1									
Anenome *indet.*															
Amphipoda *indet.*				1											
Limopontia depressa															
(A)	34	19	10	3	6	26	15	123	354	253	100	302	18	1	73
(S)	3	3	1	3	3	6	4	7	10	8	5	5	5	1	6

	P4	P5	J1	J2	J3	J4	J5	PE1	PE2	PE3	PE4	PE5
Nephtys hombergi		4	2	3	1	1	1	2	1	4	1	8
Nereis diversicolor			19	1	5	8	3	3	1		2	
Streblospio shrubsoli	4	8	106	12	4	5	5	4	4	32	668	434
Pygospio elegans											31	62
Spio martinensis									1		4	8
Phyllodoce mucosa												
Glycera spp.												
Tharyx marioni								1	7	692	80	90
Ampharete acutifrons								1		1	3	14
Melinna palmata		2								2		2
Manayunkia			3		7			6	94		4	
Pectinaria littoralis												
Tubificoides benedeni	93	120	876	569	407	51	410	303	340	11	729	334
Capitella capitata					31	74	44	28	15	21		
Heteromastus filiformis												
Hydrobia ulvae	1	9	9	2	4	2		9	14	18		10
Cerastoderma edule		2	12	6	8	3	2	3	15	6	2	10
Scrobicularia plana			5					1				
Abra prismatica												
Macoma balthica												
Ostrea edulis												
Carcinus maenas				1								
Mysid *indet*											1	
Anenome *indet.*									1	1		
Amphipoda *indet.*							9					
Limopontia depressa												
(A)	98	145	1032	604	467	540	474	370	493	869	1525	972
(S)	3	6	8	7	8	7	7	1	1	10	1	10
								1	1		1	

Number of variables	Correlation value (r)	SfG	Larval assay	Cell assay a	Cell assay b	Copper in mussel	Mortality May 95	mortality June 95	Mussel dry wt.	Mussel BCI	Mussel respiration	Mussel absorption	Mussesl consumption	Mussel clearance	Zinc in mussels	*Nephtys* width
2	0.289		*				*									
2	0.285	*	*													
2	0.276		*			*					*					
2	0.272		*										*			
2	0.257		*											*		
2	0.255	*						*								
2	0.232							*				*				
2	0.226		*									*				
2	0.218					*				*						
2	0.204				*		*									
2	0.172					*			*							
2	0.167					*										*
2	0.155						*	*								
2	0.128				*											*
2	0.113			*												*
2	0.111			*			*									
2	0.108	*			*											
2	0.104								*	*						
2	0.078	*							*							
2	0.078	*								*						
2	0.074									*	*					
2	0.074					*										
2	0.074										*					
2	0.067	*				*										
2	0.037					*										
2	0.037					*										
2	0.033															
2	0.033															
2	0.013								*							
2	0.009					*										
2	0.003						*	*								
2	0.003							*		*						
2	-0.005								*		*					
2	-0.015				*							*				
2	-0.016	*		*												
2	-0.019										*					*
2	-0.024								*			*				
2	-0.049	*														*
2	-0.05									*						
2	-0.068									*			*			
2	-0.068									*				*		
2	-0.077				*								*			
2	-0.077					*								*		
2	-0.081										*					*
2	-0.081										*		*			
2	-0.095								-						*	

Number of variables	Correlation value (r)	SfG	Larval assay	Cell assay a	Cell assay b	Copper in mussel	Mortality May 95	mortality June 95	Mussel dry wt.	Mussel BCI	Mussel respiration	Mussel absorption	Mussel consumption	Mussel clearance	Zinc in mussels	*Nephtys* width
2	-0.099														*	*
2	-0.114	*									*					
2	-0.125											*			*	
2	-0.129								*					*		
2	-0.149			*								*				
2	-0.161			*									*			
2	-0.164						*				*					
2	-0.172										*	*				
2	-0.197			*										*		
2	-0.215								*							*
2	-0.234									*						*
2	-0.239						*						*			
2	-0.239						*							*		
2	-0.248	*					*									
2	-0.248						*					*				
2	-0.338										*					*
2	-0.368								*				*			
2	-0.387	*														*
2	-0.417											*				*
2	-0.427	*											*			
2	-0.427	*												*		
2	-0.477												*	*		
2	-0.486											*	*			
2	-0.486											*		*		
2	-0.533						*									*
2	-0.555												*			*
2	-0.57	*										*				
3	0.676		*		*	*										
3	0.638		*					*								*
3	0.637		*		*				*							
3	0.637		*		*					*						
3	0.637		*		*						*					
3	0.637		*		*										*	
3	0.629				*			*		*						
3	0.612			*	*										*	
3	0.606		*	*				*								
3	0.592		*			*		*								
3	0.592		*	*							*					
3	0.592			*	*						*					
3	0.589		*		*			*								
3	0.581		*	*											*	
3	0.58		*	*	*											
3	0.58			*	*			*								
3	0.58		*				*	*								
3	0.573				*	*				*						
3	0.572		*					*	*							
3	0.572		*					*		*						

BIOENV results: Heavy metal vs. biomarkers

Number of variables	Correlation value (r)	SfG	Larval assay	Cell assay a	Cell assay b	Copper in mussel	Mortality May 95	mortality June 95	Mussel dry wt.	Mussel BCI	Mussel respiration	Mussel absorption	Mussel consumption	Mussel clearance	Zinc in mussels	*Nephtys* width
1	0.847							*							*	
1	0.797															
1	0.759										*					
1	0.71								*							
1	0.625					*										
1	0.549									*						
1	0.5			*												
1	0.499						*									
1	0.281				*											
1	0.261		*													
1	-0.01												*			
1	-0.01													*		
1	-0.025	*														
1	-0.153											*				
1	-0.193															*
2	0.951					*			*							
2	0.95							*			*					
2	0.941					*									*	
2	0.932					*					*					
2	0.905							*							*	
2	0.9			*											*	
2	0.888					*				*						
2	0.88		*												*	
2	0.876								*						*	
2	0.866			*							*					
2	0.86						*	*								
2	0.836		*								*					
2	0.814							*	*							
2	0.814							*		*						
2	0.811					*		*								
2	0.781										*				*	
2	0.779								*		*					
2	0.767							*					*			
2	0.767							*						*		
2	0.737							*				*				
2	0.734					*	*									
2	0.731		*						*							
2	0.722									*	*					
2	0.722									*					*	
2	0.714												*		*	
2	0.714													*	*	
2	0.711				*										*	
2	0.71								*	*						
2	0.708					*							*			
2	0.708					*		..						*		

Number of variables	Correlation value (r)	SfG	Larval assay	Cell assay a	Cell assay b	Copper in mussel	Mortality May 95	mortality June 95	Mussel dry wt.	Mussel BCI	Mussel respiration	Mussel absorption	Mussesl consumption	Mussel clearance	Zinc in mussels	*Nephtys* width
2	0.705	*						*								
2	0.703						*								*	
2	0.7					*						*				
2	0.697											*			*	
2	0.684						*				*					
2	0.678				*						*					
2	0.675		*			*										
2	0.662			*					*							
2	0.662			*						*						
2	0.649	*				*										
2	0.641						*		*							
2	0.641						*			*						
2	0.637		*							*						
2	0.634				*				*							
2	0.627			*				*								
2	0.624		*					*								
2	0.623				*	*										
2	0.605			*		*										
2	0.605	*													*	
2	0.59		*										*			
2	0.579										*		*			
2	0.579										*			*		
2	0.578										*	*				
2	0.57			*			*									
2	0.55				*					*						
2	0.549														*	*
2	0.541				*			*								
2	0.535	*									*					
2	0.532	*	*													
2	0.526		*				*									
2	0.513				*		*									
2	0.512		*									*				
2	0.51		*	*												
2	0.504								*			*				
2	0.493		*											*		
2	0.492	*							*							
2	0.492	*								*						
2	0.471							*								*
2	0.458								*							*
2	0.454	*					*									
2	0.454						*					*				
2	0.435			*	*											
2	0.42					*										*
2	0.414					*										*
2	0.414									*				*		
2	0.402									*		*				
2	0.402								*					*		

Number of variables	Correlation value (r)	SfG	Larval assay	Cell assay a	Cell assay b	Copper in mussel	Mortality May 95	mortality June 95	Mussel dry wt.	Mussel BCI	Mussel respiration	Mussel absorption	Mussesl consumption	Mussel clearance	Zinc in mussels	*Nephtys* width
2	0.397									*						*
2	0.373								*				*			
2	0.349	*			*											
2	0.339		*		*											
2	0.308										*					*
2	0.305		*													*
2	0.304				*							*				
2	0.283				*								*			
2	0.283				*									*		
2	0.278				*											*
2	0.244	*		*												
2	0.235			*												*
2	0.233						*						*			
2	0.233						*							*		
2	0.207			*								*				
2	0.192			*										*		
2	0.181			*									*			
2	0.107						*									*
2	-0.001	*										*				
2	-0.01												*	*		
2	-0.02	*											*			
2	-0.02	*												*		
2	-0.069											*	*			
2	-0.069											*		*		
2	-0.136												*			*
2	-0.136													*		*
2	-0.149											*				*
2	-0.229	*														*
3	0.972					*			*						*	
3	0.96					*		*		*						
3	0.95							*	*						*	
3	0.947					*		*	*							
3	0.941			*							*				*	
3	0.927		*			*					*					
3	0.927			*				*			*					
3	0.925					*			*		*					
3	0.924							*	*		*					
3	0.924					*				*					*	
3	0.924		*								*				*	
3	0.915					*		*			*					
3	0.9		*			*			*							
3	0.9		*			*				*						
3	0.9			*		*									*	
3	0.899							*				*			*	
3	0.896					*	*	*								
3	0.896					*					*				*	
3	0.891		*					*			*					

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* Articles consulted during the project but not cited in text.

This thesis is dedicated to those who I've loved but have lost,

and to she who I've gained and I cherish.